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# ANTIMICROBIAL ACTIVITY OF ANTIBODIES

## Related Applications

This application claims priority under 35 U.S.C. 119(e) to U.S. Provisional

5 Application Ser. No. 60/426,242 filed November 14, 2002, which is in corporated

herein by reference.

This application also claims priority from U.S. Application Ser. No.

10/380,905 filed March 17, 2003, which is a U.S. National Stage filing from

International Application Ser. No. PCT Application No. PCT/US01/29165 filed

10 September 17, 2001 and published in English as WO 02/022573 on March 21, 2002,

which claimed priority from U.S. Provisional Application Ser. No. 60/315,906 filed

August 29, 2001, U.S. Provisional Application Ser. No. 60/235,475 filed September

26, 2000, and U.S. Provisional Application Ser. No. 60/232,702 filed September 15,

2001, which applications.

15 This application is also related to Provisional Application Ser. No.

60/426,245 filed November 14, 2002 and to U.S. Application Ser. No. 10/714,567

filed on even date herewith.

## Government Support

20 Work contributing to this invention was supported by a grant from the

National Institutes of Health, GM43858, PO1CA277489. Accordingly, the United

States government has certain rights in the invention.

## Field of the Invention

25 The invention relates to antibody-mediated generation of reactive oxygen

species from singlet oxygen and to therapeutic compositions and methods for

treating microbial infections by using such antibodies.

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## Background

Research throughout the last century has led to a consensus as to the role of antibodies in the immune system. The essence of this consensus is that the antibody molecule does not directly kill, or otherwise generate a product that could

5      adversely affect, its target. Instead, the antibody molecule has been perceived as a binding molecule that merely tags its target or that activates other molecules or biological systems to respond to an antibody-antigen complex. Hence, antibodies themselves have been perceived as not possessing any destructive ability but as only marking foreign substances for removal by the complement cascade and/or

10     phagocytosis (Arlaud et al., Immunol. Today, 8, 106-111 (1987); Sim & Reid, Immunol. Today, 12, 307-311 (1991)).

However, antibodies with catalytic activities that can directly destroy their targets would have utility for many applications, for example, for directly killing microbes.

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## Summary of the Invention

The invention provides methods for utilizing newly discovered abilities of antibodies to produce reactive oxygen species. According to the invention, antibodies can kill microbes by converting singlet oxygen ($^1O_2^*$) into reactive

20     oxygen species. Antibodies perform such conversion without the need for any other component of the immune system, that is, without the need for the complement cascade or phagocytosis.

Hence, according to the invention, antibodies have anti-microbial activity as a result of the production of powerful reactive oxygen species, including but not

25     limited to superoxide radical ($O_2^-$), hydroxyl radical ($OH^\bullet$), hydrogen peroxide $H_2O_2$ or ozone ($O_3$). Such activity resides in antibodies and in antibody-coated mammalian leukocytes such as neutrophils.

Thus, the invention is directed to an anti-microbial composition consisting essentially of a pharmaceutically acceptable carrier and an isolated antibody that can

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bind to a microbe, wherein the antibody can generate a reactive oxygen species when singlet oxygen ($^1O_2$) is present. The anti-microbial composition can also contain a sensitizer molecule that can generate singlet oxygen ($^1O_2$). In some embodiments, such a sensitizer can generate singlet oxygen ($^1O_2$) in the presence of

5    light. Examples of sensitizer molecule include a pterin, a flavin, a hematoporphyrin, a tetrakis(4-sulfonatophenyl) porphyrin, a bipyridyl ruthenium(II) complex, a rose Bengal dye, a quinone, a rhodamine dye, a phthalocyanine, a hypocrellin, rubrocyanin, pinacyanol, allocyanin or a chlorin. Such sensitizer molecules can be attached to the antibody. In some embodiments, the antibody is a human or a

10   humanized antibody.

Reactive oxygen species generated by the antibodies of the invention include superoxide radicals, hydroxyl radicals, hydrogen peroxide, ozone and other reactive oxygen species. In some embodiments, the reactive oxygen species is ozone.

The invention also provides methods to utilize antibodies to produce reactive

15   oxygen species from singlet oxygen to treat infections, diseases and other conditions. The invention also contemplates therapeutic compositions comprising antibody compositions that can combat microbial infections. Such antibody compositions can be engineered to exhibit increased oxidative function.

For example, in some embodiments, the invention is directed to a method of

20   treating a microbial infection in a mammal that involves administering to the mammal an anti-microbial composition consisting essentially of an antibody that can bind to a microbe and a pharmaceutically acceptable carrier, wherein the antibody can generate a reactive oxygen species when singlet oxygen ($^1O_2$) is present. The composition can also contain a sensitizer molecule that can generate

25   singlet oxygen ($^1O_2$). As described above, such sensitizer molecules can be attached to the antibody.

In other embodiments, the invention is directed to a method of generating a reactive oxygen species to inhibit the growth of a microbe that involves contacting the microbe with an antibody that can bind to the microbe and a source of singlet

oxygen ($^1O_2$). In some embodiments, the source of singlet oxygen ($^1O_2$) is a sensitizer molecule. As described above, such sensitizer molecules can be attached to the antibody.

5                              **Brief Description of the Drawings**

FIG. 1 illustrates the oxygen-dependent microbicidal action of phagocytes. The interconversion of $^1O_2$ and $O_2^{\bullet-}$ is indicated.

FIG. 2 illustrates the chemical conversion steps involved in the amplex red assay. An antibody (identified as IgG in this schematic drawing) converts $^1O_2$ to

10    $O_2^{\bullet-}$, which can spontaneously form hydrogen peroxide. In the presence of horseradish peroxidase, the hydrogen peroxide deacetylates and oxidizes the amplex red substrate, thereby generating molecule that emits fluorescence at 587 nm.

FIG. 3 shows the initial time course of $H_2O_2$ production in PBS (pH 7.4) in the presence ($\square$) or absence ($\Delta$) of murine monoclonal IgG EP2-19G2 (20 μM).

15    Error bars show the range of the data from the mean.

FIG. 4 shows the fluorescent micrograph of a single crystal of murine antibody 1D4 Fab fragment after UV irradiation and $H_2O_2$ detection with the amplex red reagent.

FIG. 5A-D illustrate the time course and reaction conditions required for

20    antibody-mediated catalysis of reactive oxygen species. FIG. 5A provides a time course of $H_2O_2$ formation in PBS (pH 7.4) with hematoporphyrin (40 μM) and visible light, in the presence ($\bullet$, filled circles) or absence ($\blacklozenge$, filled diamonds) of 31127 antibody (horse IgG, 20 μM). FIG. 5B provides an initial time course of $H_2O_2$ production with hematoporphyrin (40 μM) and visible light in the presence of

25    31127 antibody (horse IgG, 6.7 μM) with no additive in PBS (pH 7.4) ($\blacksquare$, filled squares) or NaN₃ in PBS (pH 7.4) ($\bullet$, filled circles, 100 μM) or in a $D_2O$ solution of PBS (pH 7.4) ($\blacklozenge$, filled diamonds). FIG. 5C illustrates the effect of antibody protein concentration (31127, horse IgG) on the rate of $H_2O_2$ formation. FIG. 5D illustrates the effect of oxygen concentration on the rate of $H_2O_2$ generation by the 31127

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antibody (horse IgG, 6.7 μM). All points are mean values of at least duplicate experimental determinations. Error bars are the range of experimentally measured values from the mean.

FIG. 6 is a bar graph showing the measured initial rate of $H_2O_2$ formation for
5    a panel of proteins and comparison with antibodies (data from Table 1). All points are mean values of at least duplicate experimental determinations. Error bars are the range of experimentally measured values from the mean. OVA, chick-egg ovalbumin; SOD, superoxide dismutase.

FIG. 7A illustrates the rate of $H_2O_2$ formation by UV irradiation of horse
10    IgG (6.7 μM) in PBS (pH 7.4). FIG. 7B illustrates the fluorescence emission at 326 nm (excitation = 280 nm) of the horse IgG, measured simultaneously with $H_2O_2$ formation.

FIG. 8A-F illustrate $H_2O_2$ production by antibodies under various conditions.

15    FIG. 8A illustrates the production of $H_2O_2$ by immunoglobulins and non-immunoglobulin proteins. Assays were performed by near-UV irradiation (312 nm, 800 μW $cm^{-2}$) of individual antibody/protein samples (100 μL, 6.7 μM) in phosphate-buffered saline (PBS) [10 mM sodium phosphate, 150 mM NaCl (pH 7.4)] in a sealed glass vial on a transilluminator (Fischer Biotech) under ambient
20    aerobic conditions at 20EC. Aliquots (10 μL) were removed at timed intervals throughout the assay. $H_2O_2$ concentration was determined by the amplex red method. Each data point is reported as the mean ± SEM of at least duplicate measurements: ● polyclonal (poly) immunoglobulin (Ig) G, human; O poly-IgG, horse; □ poly-IgG, sheep; ∇ monoclonal (m) IgG (WD1-6G6), murine; Δ poly-IgM,
25    human; ◊ mIgG (92H2), murine; ■ β-galactosidase (β-gal); ▲ chick ovalbumin (OVA); ▼ α-lactalbumin (α-lact); ◆ bovine serum albumin (BSA).

FIG. 8B illustrates the long-term production of $H_2O_2$ by sheep poly-IgG (6.7 μM, 200 μL). Near-UV irradiation for 8 hours in PBS in a sealed well of a 96-well quartz plate. $H_2O_2$ concentration was measured as described in FIG. 8A. FIG. 8C

illustrates the effect of catalase on the antibody-catalyzed production of $H_2O_2$ over time. A solution of murine monoclonal antibody PCP-21H3 (IgG) (6.7 μM, 200 μL), was irradiated in PBS in a sealed well of a 96 well quartz plate for 510 min. The $H_2O_2$ was assayed by the amplex red assay and then destroyed by addition of

5  catalase (10 mg, 288 mU) immobilized on Eupergit C. The catalase was removed by filtration and the antibody solution re-irradiated for 420 min. Rate (0-510 min) = 0.368, μM min$^{-1}$ ($r^2 = 0.998$); rate (511-930 min) = 0.398 μM min$^{-1}$ ($r^2 = 0.987$).

FIG. 8D illustrates the effect of $H_2O_2$ concentration on the percent maximum rate of catalysis by horse poly-IgG antibody. Such a graph permits determination of

10  the $IC_{50}$ of $H_2O_2$ on the photo-production of $H_2O_2$ by horse poly-IgG. A solution of horse IgG (6.7 μM) was incubated with varying concentrations of $H_2O_2$ (0-450 μM) and the initial rate of $H_2O_2$ formation measured as described in FIG. 8A. The graph is a plot of rate of $H_2O_2$ formation versus $H_2O_2$ concentration and reveals an $IC_{50}$ of 225 μM.

15  FIG. 8E illustrates the long-term inhibition of antibody photo-production of $H_2O_2$ by $H_2O_2$ and complete re-establishment of activity after removal of $H_2O_2$. The assay involved an initial U.V. irradiation of horse poly-IgG (6.7 mM in PBS pH 7.4) in the presence of $H_2O_2$ (450 μM) for 360 min. The $H_2O_2$ was then removed by catalase (immobilized on Eupergit C) and the poly-IgG sample was re-irradiated

20  with UV light for a further 480 minutes. $H_2O_2$ formation throughout the assay was measured by the amplex red assay.

FIG. 8F illustrates the effect of catalase on $H_2O_2$ production. A solution of αβ-TCR (6.7μM, 200 μL) was irradiated as described for FIG. 8C for periods of 360, 367 and 389 min. The $H_2O_2$ generated during each irradiation was assayed and

25  destroyed as described for FIG. 8C. Rate (0-360 min) = 0.693 μM min$^{-1}$ ($r^2 = 0.962$). The curvature in the progress curve above 200 μM conforms to the expected inhibition by $H_2O_2$ (*vide infra*); rate (361-727 min) = 0.427 μM min$^{-1}$ ($r^2 = 0.987$); rate (728-1117 min) = 0.386 μM min$^{-1}$ ($r^2 = 0.991$).

FIG. 9A-B illustrate the superposition of native 4C6 Fab (light blue and pink in a color photograph) and 4C6 Fab in the presence of $H_2O_2$ (dark blue and red in a color photograph).

For FIG. 9A, the native 4C6 crystals were soaked for 3 minutes in 4 mM

5    $H_2O_2$, and immediately flash frozen for data collection at SSRL BL 9-1. The overall structural integrity of the secondary and tertiary structure is clearly preserved in the presence of $H_2O_2$ (RMSD C$\alpha$ = 0.33 Å, side chain = 0.49 Å). The RMSD was calculated in CNS.

FIG. 9B illustrates the binding of benzoic to Fab 4C6. High resolution x-ray

10    structures show that Fab 4C6 is cross-reactive with benzoic acid. Superposition of the 4C6 combining site with and without $H_2O_2$ demonstrates that even the side chain conformations within the binding site are preserved (light and dark colored side chains in a color photograph correspond to + and - $H_2O_2$ respectively). Moreover, clear electron density for the benzoic acid underscores that the binding properties of

15    Fab 4C6 remain unaltered in 4mM $H_2O_2$. The electron density map is a $2f_o$-$f_c$ sigma weighted map contoured at 1.5$\sigma$, and the figures were generated in Bobscript.

FIG. 10A shows the absorbance spectra of horse polyclonal IgG measured on a diode array HP8452A spectrophotometer, $Abs_{max}$ 280 nm.

FIG. 10B provides an action spectra of horse polyclonal IgG, between

20    wavelengths 260 and 320 nm showing maximum activity of $H_2O_2$ formation at 280 nm. The assay was performed in duplicate and involved addition of an antibody solution [6.7 µM in PBS (pH 7.4)] to a quartz tube that was then placed in a light beam produced by a xenon arc lamp and monochromator of an SLM spectrofluorimeter for 1 hour. $H_2O_2$ concentration was measured by the amplex red

25    assay.

FIG. 11A illustrates the production of $H_2O_2$ over time by tryptophan (20 µM). The conditions and assay procedures were as described in FIG. 8A.

FIG. 11B provides the effect of chloride ion on antibody-mediated photo-production of $H_2O_2$. A solution of sheep poly-IgG ■ (6.7 µM, 200 µL) or

horse poly-IgG ▲(6.7 µM, 200 µL) was lyophilized to dryness and then dissolved in either deionized water or NaCl (aq.) such that the final concentration of chloride ion was 0-160 mM. The samples were then irradiated, in duplicate, in sealed glass vials on a transilluminator (800 µW cm$^{-2}$) under ambient aerobic conditions at 20

5    EC. Aliquots (10 µL) were removed throughout the assay and the $H_2O_2$ concentration determined by the amplex red assay. The rate of $H_2O_2$ formation is plotted as the mean ±S.E.M. versus [NaCl] for each antibody sample.

FIG. 11C illustrates the effect of dialysis in EDTA-containing buffers on antibody-mediated photo-production of $H_2O_2$. The photo-production of $H_2O_2$ by

10    two antibody preparations, mouse monoclonal antibody PCP21H3 and horse polyclonal IgG, were compared before and after dialysis into PBS containing EDTA (20 mM). The conditions and assay procedures were as described in FIG. 8A. Each data point is reported as the mean ± SEM of at least duplicate measurements: [● murine mIgG PCP21H3 before dialysis; ■ murine mIgG PCP21H3 after dialysis; ▲

15    poly-IgG, horse before dialysis; ◆ poly-IgG, horse after dialysis.

FIG. 12A-F provide mass spectra illustrating oxidation of the substrate tris carboxyethyl phosphine (TCEP) with either $^{16}O$ containing $H_2O_2$ or with $^{18}O$ containing $H_2O_2$. ESI (negative polarity) mass spectra were taken of TCEP [(M-H)$^-$ 249] and its oxides [(M-H)$^-$ 265 ($^{16}O$) and (M-H)$^-$ 267 ($^{18}O$)] after oxidation with

20    $H_2O_2$.

FIG. 12A provides the mass spectrum of TCEP and its oxides after irradiation of sheep poly-IgG (6.7/µM) under $^{16}O_2$ aerobic conditions in $H_2^{18}O$ (98 % $^{18}O$) PB. A mix of $^{16}O$ containing TCEP (larger peak at 265) and $^{18}O$ containing TCEP (smaller peak at 267) is produced.

25    FIG. 12B provides the mass spectrum of TCEP and its oxides after irradiation of sheep poly-IgG (6.7 µM) under enriched $^{18}O_2$ (90 % $^{18}O$) aerobic conditions in $H_2^{16}O$ PB. A mix of $^{16}O$ containing TCEP (smaller peak at 265) and $^{18}O$ containing TCEP (larger peak at 267) is produced.

FIG. 12C provides the mass spectrum of TCEP and its oxides after irradiation of the poly-IgG performed under $^{16}O_2$ aerobic concentration in $H_2^{16}O$ PB. The assay conditions and procedures were as described in the methods and materials (Example II) with the exception that $H_2^{16}O$ replaced $H_2^{18}O$. Only $^{16}O$ containing

5      TCEP (large peak at 265) is observed.

FIG. 12D provides the mass spectrum of TCEP and its oxides after irradiation of sheep poly-IgG (6.7 µM) and $H_2^{16}O_2$ (200 µM) under anaerobic (degassed and under argon) conditions in $H_2^{18}O$ PB for 8 hours at 20EC. Addition of TCEP was as described in the methods and materials (Example II). . Only $^{16}O$

10      containing TCEP (large peak at 265) is observed.

FIG. 12E provides the mass spectrum of TCEP and its oxides after irradiation of 3-methylindole (500 µM) under $^{16}O_2$ aerobic conditions in $H_2^{18}O$ PB. Only $^{16}O$ containing TCEP (large peak at 265) is observed. The assay conditions and procedures were as described in the methods and materials (Example II) with

15      the exception that size-exclusion filtration was not performed because 3-methyl indole is of too low molecular weight. Therefore, TCEP was added to the 3-methyl indole-containing PB solution.

FIG. 12F provides the mass spectrum of TCEP and its oxides after irradiation of β-gal (50 µM) under $^{16}O_2$ aerobic conditions in $H_2^{18}O$ PB. Only $^{16}O$

20      containing TCEP (large peak at 265) is observed. Assay conditions and procedures are as described in the methods and materials (Example II).

FIG. 13A-B show the Xe binding sites in antibody 4C6 as described in materials and methods (Example II).

FIG. 13A provides a standard side view of the Cα trace of Fab 4C6 with the

25      light chain in pink and the heavy chain in blue in a color photograph. Three bound xenon atoms (green in a color photograph) are shown with the initial $F_o$-$F_c$ electron density map contoured at 5 σ.

FIG. 13B provides an overlay of Fab 4C6 and the 2C αβ TCR (PDB/TCR) around the conserved xenon site 1. The backbone $C_α$ trace of $V_L$ (pink in a color

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photograph) and side chains (yellow in a color photograph) and the corresponding $V_\alpha$ of the 2C $\alpha\beta$ TCR (red and gold in a color photograph) are superimposed (FIG. generated using Insight2000).

FIG. 14A-D illustrate the killing of bacteria by antibodies.

5        FIG. 14A provides a bar-graph showing the survival of *E. coli* XL1- blue and O112a,c strains under different experimental conditions. Survival is reported as recovered colony forming units (CFUs) as a percent of the CFUs at the start of the experiment ($t = 0$ min). Hatched bars and open bars correspond to the same experimental conditions except that the open bar groups (2, 4, 6, 8, 10 and 12) were

10       exposed to visible light (2.7 mWcm$^{-2}$) for 60 min, whereas the hatched bar groups (1, 3, 5, 7, 9 and 11) were placed in the dark for 60 min. The bacterial cell density was about $10^7$ cells/mL. Each data point reported is the mean $\pm$ S.E.M. ($n=6$) of *E. coli* XL1- blue (groups 1-6) and O112 a,c (groups 7-12) under the following conditions. Groups 1-2 XL1-blue cells in PBS, pH 7.4 at 4 °C. Groups 3-4 HPIX

15       (40 µM), XL1-blue cells in PBS, pH 7.4 at 4 °C. Groups 5-6 XL1-blue-specific monoclonal antibody (25D11, 20 µM), hematoporphyrin IX (40 µM), XL1-blue cells in PBS, pH 7.4 at 4 °C. Groups 7-8 O112 a,c cells in PBS, pH 7.4 at 4 °C. Groups 9-10 HPIX (40 °M), O112a,c cells in PBS, pH 7.4 at 4 °C. Groups 11-12 O112a,c-specific monoclonal antibody (15404, 20 µM), hematoporphyrin IX (40

20       µM), O112a,c cells in PBS, pH 7.4 at 4 °C.

FIG. 14B graphically illustrates the effect of antibody concentration on the survival of *E. coli* O112a,c. The antibody employed was an O112a,c-specific monoclonal antibody, 15404. Each data point reported is the mean value $\pm$ S.E.M ($n=3$). The concentration of 15404 antibody that corresponds to killing of 50 % of

25       the cells (EC$_{50}$) was 81 $\pm$ 6 nM.

FIG. 14C graphically illustrates the effect of irradiation time on the bactericidal action of *E. coli* XL1-blue-specific murine monoclonal antibody 12B2. The graph provides irradiation time (2.7 mW cm$^{-2}$) *versus* survival of *E. coli* XL1-blue in the presence of hematoporphyrin IX (40 µM) and 12B2 (20 µM). Each data

point reported is the mean value ± S.E.M ($n$=3). The time of irradiation that corresponds to killing of 50 % of the cells was 30 ± 2 min.

FIG. 14D illustrates the dependence of antibody driven bactericidal action on hematoporphyrin IX concentration. The antibody employed was the *E. coli*

5    XL1-blue-specific murine monoclonal antibody 25D11. The graph provides survival of *E. coli* XL1-blue *versus* exposure to a range of hematoporphyrin IX concentrations. The following conditions were employed: XL1-blue cells in PBS, pH 7.4 at 4 °C in the dark, 60 min (▲). XL1- blue cells in PBS, pH 7.4 at 4 °C in white light (2.7 mW cm$^{-2}$) (Δ). 25D11 (20 μM), XL1- blue cells in PBS, pH 7.4 at 4

10   °C in the dark, 60 min (♦). 25D11 (20 μM), XL1-blue cells in PBS, pH 7.4 at 4 °C in white light (2.7 mW cm$^{-2}$) for 60 min (◊).

FIG. 15 provides an electron micrograph of an *E. coli* O112a,c cell after exposure to antigen-specific murine monoclonal IgG (15404, 20 μM), hematoporphyrin IX (40 μM) in PBS and visible light for 1 h at 4 °C (< 5 % viable).

15   To visualize the sites of antibody attachment gold-labeled goat anti-mouse antibodies were added after completion of the bactericidal assay. The potency of the bactericidal activity of antigen non-specific antibodies was observed to be very similar to antigen-specific antibodies. Typically 20 μM of antibody (non-specific) was > 95 % bactericidal in the assay system.

20   FIG. 16A-C provide electron micrographs of *E. coli* XL-1 blue cells after exposure to non-specific murine monoclonal IgG antibodies (84G3, 20 μM), hematoporphyrin IX (40 μM) in PBS and visible light for 1 h at 4 °C (1 % viable). The arrows in FIG. 16A point toward the preliminary separation of the cell membrane from the cytoplasmic contents. FIG. 16D provides an electron

25   micrograph of serotype *E. coli* O112a,c after exposure to antigen-specific murine monoclonal IgG (15404, 10 μM), hematoporphyrin IX (40 μM) in PBS and visible light for 1 h at room temperature (< 5 % viable). Gold-labeling was performed using procedures available in the art.

FIG. 17A illustrates the effect of catalase on the bactericidal action of antibodies against $E.coli$ XL1-blue [reported as recovered colony forming units (CFUs) as a percent of the CFUs at the start of the experiment ($t = 0$ min)]. Catalase converts $H_2O_2$ to water ($H_2O$) and molecular oxygen ($O_2$). Each group was

5  irradiated with white light (2.7 mW $cm^{-2}$) for 60 min at 4 °C. The bacterial cell density was ~ $10^7$ cells/mL. The experimental groups (1- 7) were treated as follows: Group 1 $E. coli$ XL1-blue cells and hematoporphyrin IX (40 $\mu$M) in PBS (pH 7.4). Group 2 $E. coli$ XL1-blue cells and non-specific murine monoclonal antibody 84G3 (20 $\mu$M) in PBS (pH 7.4). Group 3 $E. coli$ XL1-blue cells, hematoporphyrin IX (40

10  $\mu$M) and monoclonal antibody 84G3 (20 $\mu$M) in PBS (pH 7.4). Group 4 $E. coli$ XL1-blue cells, hematoporphyrin IX (40 $\mu$M), monoclonal antibody 84G3 (20 $\mu$M) and catalase (13mU/mL) in PBS (pH 7.4). Group 5 $E. coli$ XL1-blue cells and specific rabbit polyclonal antibody (20 $\mu$M) in PBS (pH 7.4). Group 6 $E. coli$ XL1-blue cells, hematoporphyrin IX (40 $\mu$M) and specific rabbit polyclonal antibody (20

15  $\mu$M) in PBS (pH 7.4). Group 7 $E. coli$ XL1-blue cells, hematoporphyrin IX (40 $\mu$M), specific rabbit polyclonal antibody (20 $\mu$M) and catalase (13mU/mL) in PBS (pH 7.4). Each point is reported as the mean value ± S.E.M. of multiple experiments ($n$=6). The symbol ** denotes a $p$ value of < 0.01 relative to controls at the same time point. No bactericidal activity was observed in any of the dark

20  controls (data not shown).

FIG. 17B illustrates the concentration dependent toxicity of $H_2O_2$ on the viability of $E. coli$ XL1-blue (■) and O112a,c (▲) serotypes. The vertical hatched line is the concentration of $H_2O_2$ expected to be generated by antibodies during a 60 min incubation using the conditions described above for FIG. 14 and in Hofman et

25  al., $Infect.$ $Immun.$ **68**, 449 (2000). The value of 35 ± 5 $\mu$M $H_2O_2$ is the mean value determined from at least duplicate assays of twelve different monoclonal antibodies.

FIG. 18 illustrates the progress of photo-production of isatin sulfonic acid **2** from indigo carmine **1** (1 mM) during u.v. irradiation (312 nm, 0.8 mW $cm^{-2}$) of antibodies in PBS (pH 7.4) in the presence and absence of catalase. Steinbeck et al.,

*J. Biol. Chem.* **267**, 13425 (1992). Each point is reported as the mean ± S.E.M. of at least duplicate determinations. Linear regression analysis was performed with Graphpad Prism v.3.0 software. The rate of formation of isatin sulfonic acid **2** ($v$) was observed under the following conditions: Sheep polyclonal IgG (20 μM) (•) $v$

5    = 34.8 ± 1.8 nM/min; Murine monoclonal antibody 33F12 (20 μM) (□) $v$ = 40.5 ± 1.5 nm/min; Sheep polyclonal IgG (20 μM) and soluble catalase (13 mU/mL) (Δ) $v$ = 33.5 ± 2.3 nM/min; Murine monoclonal antibody 33F12 (20 μM) and soluble catalase (13 mU/mL) (∇) $v$ = 41.8 ± 1.2 nM/min.

FIG. 19A-C provides electrospray ionization (negative polarity) mass

10    spectra of isatin sulfonic acid **2** [(MH)- 226, (M-H)- 228 (^{18}O) and (M-H)- (2 x ^{18}O)] produced during the oxidation of indigo carmine **1** (1 mM) in $H_2$ [18]O (> 95 % [18]O) phosphate buffer (PB, 100 mM, pH 7.4) at room temperature under various conditions. FIG. 19A provides the mass spectrum of isatin sulfonic acid **2** produced during the oxidation of indigo carmine **1** by chemical ozonolysis (600 μM in PB)

15    for 5 min. The [18]O is the dark circle and the [16]O is the open circle. FIG. 19B provides the mass spectrum of isatin sulfonic acid **2** produced during the oxidation of indigo carmine **1** by irradiation with white light (2.7 mW cm$^{-2}$), hematoporphyrin IX (40 μM) and sheep poly-IgG (20 μM) for 4 h. FIG. 19C provides the mass spectrum of isatin sulfonic acid **2** produced during the oxidation of indigo carmine **1**

20    by irradiation of hematoporphyrin IX (40 μM) with white light (2.7 mW cm$^{-2}$) for 4 h.

FIG. 20A illustrates the time course of oxidation of indigo carmine **1** (30 μM) (▲)and formation of **2** (■) by human neutrophils (PMNs, 1.5 x 10$^7$ cell/mL) activated with phorbol myristate (1 μg/mL) in PBS (pH 7.4) at 37 °C. No oxidation

25    of indigo carmine **1** occurs with PMNs that are not activated (data not shown). Neutrophils were prepared as previously described. Hypochlorous acid (HOCl) is an oxidant known to be produced by neutrophils. In our hands, NaOCl (2 mM) in

PBS (pH 7.4) oxidizes **1** (100 μM) but does not cleave the double bond of **1** to yield isatin sulfonic acid **2**.

FIG. 20B illustrates the negative-ion electrospray mass spectrum of the isatin sulfonic acid **2** produced during the oxidation of indigo carmine **1** by activated

5     human neutrophils, under the conditions described in FIG. 20A.

FIGs. 21A-B provide bar graphs illustrating the bactericidal activity of the *S. typhimurium*-specific antibody 6B5. The mean colony forming units ($n$=3) are shown at time zero ($t$= 0, blue) and at after one hour ($t$= 1 h, magenta) incubation of live *S. typhimurium* with the 6B5 antibody preparation under different conditions.

10    FIG. 21A shows the results of experiments performed with white light irradiation (1.5 mWcm$^{-2}$ light flux). The assays contained: *Salmonella* cells alone; *Salmonella* cells and hematoporphyrin IX (HP) (120 $\mu$M); *Salmonella* cells and 6B5 antibodies (40 $\mu$M); *Salmonella* cells, 6B5 antibodies (5 $\mu$M) and hematoporphyrin IX (120 $\mu$M); *Salmonella* cells, 6B5 antibodies (10 $\mu$M) and hematoporphyrin IX

15    (120 $\mu$M); *Salmonella* cells, 6B5 antibodies (20 $\mu$M) and hematoporphyrin IX (120 $\mu$M); *Salmonella* cells, 6B5 antibodies (30 $\mu$M) and hematoporphyrin IX (120 $\mu$M); *Salmonella* cells, 6B5 antibodies (40 $\mu$M) and hematoporphyrin IX (120 $\mu$M).

FIG. 21B shows the results of experiments performed in the dark (zero light flux). The assays contained: *Salmonella* cells alone; *Salmonella* cells and

20    hematoporphyrin IX (120 $\mu$M); *Salmonella* cells and 6B5 antibodies (40 $\mu$M); *Salmonella* cells, 6B5 antibodies (5 $\mu$M) and hematoporphyrin IX (120 $\mu$M); *Salmonella* cells, 6B5 antibodies (10 $\mu$M) and hematoporphyrin IX (120 $\mu$M); *Salmonella* cells, 6B5 antibodies (20 $\mu$M) and hematoporphyrin IX (120 $\mu$M); *Salmonella* cells, 6B5 antibodies (30 $\mu$M) and hematoporphyrin IX (120 $\mu$M);

25    *Salmonella* cells, 6B5 antibodies (40 $\mu$M) and hematoporphyrin IX (120 $\mu$M).

## Detailed Description of the Invention

The present invention concerns the discovery that antibodies, as a class of molecules, have the ability to convert singlet oxygen to reactive oxygen species. According to the invention, such reactive oxygen species can kill microbes.

5    Examples of reactive oxygen species generated by antibodies include, but are not limited to ozone ($O_3$), superoxide radical ($O_2^-$), hydrogen peroxide ($H_2O_2$) or hydroxyl radical ($OH^\bullet$).

The ability of antibodies to convert singlet oxygen to reactive oxygen species, regardless of source or antigenic specificity of the antibody, links the

10    previously appreciated binding properties of antibodies with an ability to destroy their target. The present invention therefore provides methods for inhibiting microbial growth that involve contacting a microbe with an antibody that can generate a reactive oxygen species.

15    **Definitions**

Abbreviations: (HP) hematoporphyrin; (PBS) phosphate buffered saline; (OVA) chick-egg ovalbumin; (SOD) superoxide dismutase; (PO) peroxidase enzymes; (phox) phagocyte oxidase; (HRP) horseradish peroxidase; (MS) mass spectroscopy; (AES) ICP-atomic emission spectroscopy; (MS) mass-spectral, (QC)

20    quantum chemical.

The term "agent" herein is used to denotes a chemical compound, a mixture of chemical compounds, a biological macromolecule, or an extract made from biological materials such as bacteria, plants, fungi, or animal (particularly mammalian) cells or tissues. Agents are evaluated for potential activity as antibody

25    or neutrophil modulatory agents by screening assays described herein.

The terms "effective amount," "effective reducing amount," "effective ameliorating amount", "effective bactericidal amount," "effective tissue injury inhibiting amount", "therapeutically effective amount" and the like terms as used herein are terms to identify an amount sufficient to obtain the desired physiological

effect, e.g., treatment of a condition, disorder, disease and the like or reduction in symptoms of the condition, disorder, disease and the like. Such an effective amount of an antibody in the context of therapeutic methods is an amount that results in reducing, reversing, ameliorating, or inhibiting a microbial infection.

5    An "engineered antibody molecule" is a polypeptide that has been produced through recombinant techniques. Such molecules can include a reactive center that can catalyze the production of at least one reactive oxygen species from singlet oxygen. Such engineered antibody molecules may have a reactive indole contained within a polypeptide structure. The indole of such a molecule may be present as a

10   tryptophan residue. Engineered antibody molecules may also contain non-natural amino acids and linkages as well as peptidomimetics. Engineered antibody molecules also include antibodies that are modified to eliminate the reaction center such that they are substantially unable to generate reactive oxygen species.

As used herein, the term "epitope" means any antigenic determinant on an

15   antigen to which the paratope of an antibody binds. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three-dimensional structural characteristics, as well as specific charge characteristics. Antigens can include polypeptides, fatty acids, lipoproteins, lipids, chemicals, hormones and the like. In

20   some embodiments, antigens include, but are not limited to, proteins from microbes such as bacteria or viruses such as human immunodeficiency virus, influenza virus, herpesvirus, papillomavirus, human T-cell leukemia virus and the like. In other embodiments, antigens include, but are not limited to, proteins expressed on cancer cells such as lung cancer, prostate cancer, colon cancer, cervical cancer,

25   endometrial cancer, bladder cancer, bone cancer, leukemia, lymphoma, brain cancer and the like. Antigens of the invention also include chemicals such as ethanol, tetrahydrocanabinol, LSD, heroin, cocaine and the like.

The term "modulate" refers to the capacity to either enhance or inhibit a functional property of an antibody or engineered antibody molecule of the

invention. Such modulation may increase or decrease production of at least one reactive oxygen species by the antibody, neutrophil or engineered antibody molecule.

A "non-natural" amino acid includes D-amino acids as well as amino acids

5   that do not occur in nature, as exemplified by 4-hydroxyproline, $\gamma$-carboxyglutamate, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine and other such amino acids and imino acids.

The term "peptidomimetic" or "peptide mimetic" describes a peptide analog, such as those commonly used in the pharmaceutical industry as non-peptide

10  drugs, with properties analogous to those of the template peptide. (Fauchere, J., Adv. Drug Res., 15: 29 (1986) and Evans et al., J. Med. Chem., 30:1229 (1987)). Generally, peptidomimetics are structurally similar to a paradigm polypeptide (i.e., a polypeptide that has a biochemical property or pharmacological activity), but have one or more peptide linkages optionally replaced by a linkage such as, --

15  $CH_2NH$--, --$CH_2S$--, --$CH_2$--$CH_2$--, --$CH=CH$-- (cis and trans), --$COCH_2$--, --$CH(OH)CH_2$--, and --$CH_2SO$--, by methods known in the art. Advantages of peptide mimetics over natural polypeptide embodiments may include more economical production, greater chemical stability, altered specificity, reduced antigenicity, and enhanced pharmacological properties such as half-life, absorption,

20  potency and efficacy.

As used herein, the terms "pharmaceutically acceptable," "physiologically tolerable" and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that such materials are capable of administration to or upon a mammal without the

25  production of undesirable physiological effects such as nausea, dizziness, gastric upset and the like.

The terms "protein" and "polypeptide" are used to describe a native protein, a peptide, a protein fragment, or an analog of a protein or polypeptide. These terms may be used interchangeably.

As used herein the term "reactive oxygen species" means antibody-generated oxygen species. These reactive oxygen species can possess one or more unpaired electrons or are otherwise reactive because they are readily react with other molecules. Such reactive oxygen species include but are not limited to

5      superoxide free radicals, hydrogen peroxide, hydroxyl radical, peroxyl radical, ozone and other short-lived trioxygen adducts that have the same chemical signature as ozone.

## Catalytic Activity of Antibodies

10     According to the invention, antibodies, regardless of source or antigenic specificity, can convert singlet oxygen into reactive oxygen species such as to ozone ($O_3$), superoxide radical ($O_2^-$), hydrogen peroxide ($H_2O_2$) or hydroxyl radical ($OH^\bullet$). Such enzymatic action, joined with the high affinity targeting capabilities of antibodies, makes them into singular entities that can effectively destroy their

15     targets. The antibody is therefore more properly perceived to be a remarkable adaptor molecule, having evolved both targeting and catalytic functions that place it at the frontline of the vertebrate defense against foreign invaders.

The ability to produce reactive oxygen species from singlet oxygen is present in intact immunoglobulins and well as in antibody fragments such as Fab,

20     F(ab')$_2$ and Fv fragments (see examples). This activity does not reside in other molecules, including RNaseA, superoxide dismutase, and Bowman-Birk inhibitor protein that can be oxidized (example I and Table 1). Also, the activity is not associated with the presence of disulfides in a molecule, even though such disulfides are sufficiently electron rich that they can be oxidized (Bent et al., J. Am.

25     Chem. Soc., 87:2612-2619 (1975)).

The ability of an antibody to generate a reactive oxygen species from singlet oxygen is abolished if the antibody is denatured. This indicates that the three dimensional structure of the antibody is relevant to the reduction process used to generate reactive oxygen species.

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The ability to produce reactive oxygen species in an efficient and long term manner from singlet oxygen is present in immunoglobulins and in the T-cell receptor (example II, FIG. 1F). The T-cell receptor shares structural similarities with antibodies, including the arrangement immunoglobulin fold domains (Garcia

5      et al., Science, 274:209 (1996)). However, possession of this structural motif does not appear necessary to confer a reactive oxygen species-generating ability on proteins. $\beta_2$-macroglobulin, a member of the immunoglobulin superfamily having this structural motif, does not generate hydrogen peroxide (Welinder et al., Mol. Immunol., 28:177 (1991)).

10     Structural studies also indicate that a conserved tryptophan residue found in T-cell receptors resides in a domain similar to that found in antibodies. While antibodies and T-cell receptors both have such a tryptophan. $\beta_2$-Macroglobulin, which lacks this conserved tryptophan residue, does not have the ability to generate ozone, superoxide or hydrogen peroxide. The sequence and structure surrounding

15     this tryptophan residue is highly conserved between antibodies and T-cell receptors, indicating that those surrounding structures may also play a role in allowing catalysis of singlet oxygen to hydrogen peroxide, ozone, and/or superoxide.

The discovery of the bactericidal activity of antibodies in the presence of

20     $^1O_2^*$ is the first direct evidence that they can destroy their antigenic targets in the absence of complement or phagocytes. This is the first evidence that antibodies may play a role in host defense against bacterial or microbial infection.

## Endogenous Production of Singlet Oxygen

25     The role of the newly discovered chemical potential of antibodies *in vivo* is dependent on the availability of the key substrate $^1O_2^*$. However, $^1O_2^*$ is produced during a variety of physiological events and is available *in vivo*. See J. F. Kanofsky *Chem.-Biol. Interactions* **70**, 1 (1989) and references therein. For example, $^1O_2^*$ is produced including reperfusion. X. Zhai and M. Ashraf *Am. J. Physiol.* **269** (*Heart*

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*Circ. Physiol.* **38**) H1229 (1995). Also, $^1O_2$* is produced in neutrophil activation during phagocytosis. J. R. Kanofsky, H. Hoogland, R. Wever, S. J. Weiss *J. Biol. Chem.* **263**, 9692 (1988); Babior et al., Amer. J. Med., 109:33-34 (2000). Singlet oxygen ($^1O_2$) also results from irradiation by light of metal-free porphyrin

5      precursors that are present in the skin of porphyria sufferers.

Moreover, the substrate $^1O_2$* is generated by phagocytosis or reperfusion in amounts that are sufficient for antibodies to produce detectable levels of reactive oxygen species. For example, the volume of the phagosome is approximately 1.0 x $10^{-15}$ liters. Hence, the reactions identified herein need not be highly efficient

10     because only a few hundred molecules comprise micromolar concentrations in such a small volume. In fact, the concentration of $^1O_2$* has been calculated to be as high as a molar concentration within the phagosome. E. P. Reeves *et al.*, *Nature* **416**, 291 (2002). The same estimates can be made regarding the number of antibody molecules from titrations with bacteria and fluorescently-labeled antibodies and the

15     immuno-gold studies (Fig. 2). These analyses suggest that there are about $10^5$ antibody molecules bound to each bacterium and such amounts would correspond to a millimolar antibody concentration within the phagosome. Thus, by even the most conservative of estimates, the concentrations of $^1O_2$* and antibody within the phagosome far exceed those used in the illustrative examples provided here.

20     Singlet molecular oxygen ($^1O_2$) is also generated during microbicidal processes in both direct and indirect ways. Singlet molecular oxygen ($^1O_2$) is generated directly, for example, via the action of flavoprotein oxidases (Allen, R. C., Stjernholm, R. L., Benerito, R. R. & Steele, R. H., eds. Cormier, M. J., Hercules, D. M. & Lee, J. (Plenum, New York), pp. 498-499 (1973); Klebanoff, S.

25     J. in The Phagocytic Cell in Host Resistance (National Institute of Child Health and Human Development, Orlando, FL) (1974)). Alternatively, $^1O_2$ can be generated indirectly during microbicidal processes such as the nonenzymatic disproportionation of $O_2^{\bullet-}$ in solutions at low pH, like those found in the phagosome (Stauff, J., Sander, U. & Jaeschke, W., Chemiluminescence and

Bioluminescence, eds., Williams, R. C. & Fudenberg, H. H. (Intercontinental

Medical Book Corp., New York), pp. 131-141 (1973); Allen, R. C., Yevich, S. J.,

Orth, R. W. & Steele, R. H., Biochem. Biophys. Res. Commun., 60, 909-917

(1974)).

5      Because $^1O_2$ is so highly reactive, it was previously considered to be an

endpoint in the cascade of oxygen-scavenging agents. However, it has been found

that antibodies, as a class of proteins, have the ability to intercept $^1O_2$ and

efficiently reduce it to reactive oxygen species, thus offering a mechanism by

which $^1O_2$ can be rescued and recycled during phagocyte action, and thereby

10    potentiating the microbial action of the immune system.


**Therapeutic Methods**

      The invention provides methods for the production of reactive oxygen

species when their production is warranted, such as for inhibiting microbial

15    infection, in promoting wound healing, lysing bacteria, eliminating viruses,

targeting cancer cells for oxidant-induced lysis and the like processes. For

example, the invention provides antibody mediated generation of reactive oxygen

species to supplement the local concentration of superoxide concentration

generated by phagocytic neutrophils and to combat a bacterial infection or viral

20    infection. The reactive oxygen species acts as an anti-microbial agent destroying

the bacteria, viruses or other microbes. Thus, to enhance this process, one would

use the method of this invention to provide an antibody composition to the area to

cause an increase in the local concentration of reactive oxygen species.

      A.     Providing Antibody Activity

25    Therapeutic methods contemplated by the invention that are based on using

an antibody that can generate reactive oxygen species include 1) inhibiting

proliferation of a microbe, or targeting and killing a microbe in a patient where the

antibody recognizes and immunoreacts with an antigen expressed on the microbe,

2) inhibiting proliferation of a cancer cell, or targeting and killing a cancer cell in a

patient where the antibody recognizes and immunoreacts with an antigen expressed on the cancer cell, 3) inhibiting tissue injury associated with neutrophil mediated inflammation in a subject, for example where the inflammation results from a bacterial infection or when the subject has an autoimmune disease, 4) enhancing

5    the bactericidal effectiveness of a phagocyte in a subject, 5) promoting wound healing in a subject having a open wound where the oreactive oxygen species stimulates fibroblast proliferation and/or the immune response further includes lymphocyte proliferation, 6) stimulating cell proliferation, such as stimulating fibroblast proliferation in a wound in a subject, and similar situations.

10    In some embodiments, the invention provides therapeutic methods for treating microbial infections and other diseases that benefit from enhanced production of a reactive oxygen species such as a superoxide radical, hydroxyl radical, ozone or hydrogen peroxide. Such methods can employ any antibody to generate reactive oxygen species in a situation where the production of such a

15    reactive oxygen species is warranted. In some embodiments, the specifically targeted to bind to the microbe, so that production of reactive oxygen species is localized.

Moreover, such methods can employ an antibody that has been engineered to generate increased levels of reactive oxygen species, for example, because the

20    antibody has an additional reactive site for converting singlet oxygen to reactive oxygen species. The use of engineered antibody molecules having more than two reductive centers compared to a non-engineered antibody having the two conserved tryptophan residues is warranted when enhanced production of a reactive oxygen species such as a superoxide radical, hydroxyl radical, ozone or hydrogen peroxide

25    is needed.

In still further aspects, the antibody is a recombinant antibody that is provided as described herein or, alternatively, is expressed from an expression vector delivered to the cell. The expression vector in this context can also express a sensitizer molecule (see below).

The minimum requirement for generating a reactive oxygen species by an antibody is the presence of oxygen, i.e., aerobic conditions are generally required. The biological conversion of singlet oxygen to reactive oxygen species occurs in light, including visible light, infrared light and under ultraviolet irradiation

5      conditions. When visible light conditions are employed, the production of singlet oxygen can be enhanced using other molecules that can provide a source of singlet oxygen. Molecules that generate singlet oxygen include molecules that generate singlet oxygen without the need for other factors or inducers as well as "sensitizer" molecules that can generate singlet oxygen after exposure to an inducer. Examples

10    of molecules that can generate singlet oxygen without the need for other factors or inducers include, but are not limited to, endoperoxides. In some embodiments, the endoperoxide employed can be an anthracene-9,10-dipropionic acid endoperoxide. Examples of sensitizer molecules include, but are not limited to, pterins, flavins, hematoporphyrins, tetrakis(4-sulfonatophenyl)porphyrin, bipyridyl ruthenium(II)

15    complexes, rose Bengal dyes, quinones, rhodamine dyes, phthalocyanines, hypocrellins, rubrocyanins, pinacyanols or allocyanines.

Sensitizer molecules can be induced to generate singlet oxygen when exposed to an inducer. One such inducer is light. Such light can be visible light, ultraviolet light, or infrared light, depending upon the type and structure of the

20    sensitizer.

The invention further contemplates the therapeutic use of an antibody to create ozone, superoxide, hydroxyl radical or hydrogen peroxide in an environment where such reactive oxygen species are needed or are substantially absent. In one embodiment, the invention contemplates a method for inhibiting the growth of a

25    microbe where the microbe is contacted with a composition including an antibody able to generate such a reactive oxygen species from singlet oxygen. The method is successful when nonspecific or immunospecific (antigen binding), whole or fragment antibodies are used. Such antibody fragments include single chain antibodies as well as the engineered molecules and antibodies described herein.

30    However, when localized activity against a microbe is desired, the antibody can be specific for an antigen associated with the microbe. For example, the antibody can

bind selectively to an antigen on the surface of the microbe.

The antibody composition can be delivered *in vivo* to a subject with a microbial infection or other disease or condition that may benefit from exposure to a reactive oxygen species. Preferred *in vivo* delivery methods include

5     administration intravenously, topically, by inhalation, by cannulation, intracavitally, intramuscularly, transdermally, subcutaneously or by liposome containing the antibody.

Exemplary concentrations of antibody at the cell surface range from 0.01 to 50 micromolar. However, the concentration may vary depending on the desired

10     outcome where the amount of antibody provided is that amount of antibody that is sufficient to obtain the desired physiological effect, i.e., the generation of a reactive oxygen species such as hydrogen peroxide, ozone, a superoxide radical or a derivative oxidant thereof to generate oxidative stress. Dosing and timing of the therapeutic treatments with antibody compositions are compatible with those

15     described for antioxidants below.

The methods of the invention further contemplate exposing an antibody or antibody-antigen complex to irradiation with ultraviolet, infrared or visible light in the method of generating antibody-mediated reactive oxygen species such as hydrogen peroxide, ozone, superoxide radicals or derivative oxidants thereof.

20     To enhance the production of a reactive oxygen species, a reactive oxygen species-generating amount of a sensitizer, for example, a photosensitizer, can be utilized in the therapeutic methods described herein. As defined herein, a sensitizer is any molecule that induces or increases the concentration of singlet oxygen. Sensitizers can be used in the presence of irradiation, the process of which includes

25     exposure to ultraviolet, infrared or visible light for a period sufficient to activate the sensitizer. Exemplary exposure times and conditions are described in the examples.

A reactive oxygen species-generating amount of sensitizer is the amount of sensitizer that is sufficient to obtain the desired physiological effect, e.g.,

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generation of a reactive oxygen species such as superoxide, ozone or hydrogen peroxide from singlet oxygen, mediated by an antibody in any situation where the presence of such reactive oxygen species and the derivatives thereof is warranted. In some embodiments, a sensitizer is conjugated to the antibody. An antibody conjugated to a sensitizer is generally capable of binding to a antigen, i.e., the antibody retains an active antigen binding site, allowing for antigen recognition and complexing to occur.

Exemplary sensitizers include but are not limited to pterins, flavins, hematoporphyrin, tetrakis(4-sulfonatophenyl)porphyrin, bipyridyl ruthemium(II) complexes, rose bengal dye, quinones, rhodamine dyes, phtalocyanine, and hypocrellins.

In a further embodiment, generation of a reactive oxygen species is enhanced by administering a means to enhance production of singlet oxygen. Reduced singlet oxygen is the source of reactive oxygen species such as hydrogen peroxide, ozone, superoxide radicals or derivative oxidants thereof. One means to enhance production of singlet oxygen is a prodrug that includes any molecule, compound, or reagent that is useful in generating singlet oxygen. Such a prodrug is administered with, or at a time subsequent to, the administering or contacting of an antibody with a desired target cell, tissue or organ as described herein. When a prodrug is administered after antibody administration, the antibody has already had an opportunity to immunoreact with its target antigen and may have formed an antibody-antigen complex. A means to enhance production of singlet oxygen can then enhance the generation of reactive oxygen species such as hydrogen peroxide, ozone, superoxide radicals or derivative oxidants thereof, at the site of antibody-antigen recognition. This embodiment has particular advantages, for example, the ability to create increased local accumulation of therapeutically desirable superoxide, ozone or hydrogen peroxide at a desired site or location.

A preferred prodrug is endoperoxide, for example, at a concentration of about 1 micromolar to about 50 micromolar. A preferred concentration of

endoperoxide to achieve at the antibody-antigen complex site is about 10 micromolar.

An antigenic target of the antibodies of the invention can be any antigen known or available to one of skill in the art. The antigen can be any antigen that is

5    present on or in a cell, tissue or organ where the presence of reactive oxygen species and the antibody mediated process of producing it is warranted. The antigen can be in solution, for example, in extracellular fluids. An antigen can be, for example, a protein, a peptide, a fatty acid, a low density lipoprotein, an antigen associated with inflammation, a cancer cell antigen, a bacterial antigen, a viral

10   antigen or a similar molecule.

Cells on which antigens are associated include but are not limited to microbial, endothelial, interstitial, epithelial, muscle, phagocytic, blood, dendritic, connective tissue and nervous system cells.

Hence, for example, infections of the following target microbial organisms

15   can be treated by the antibodies of the invention: *Aeromonas* spp., *Bacillus* spp., *Bacteroides* spp., *Campylobacter* spp., *Clostridium* spp., *Enterobacter* spp., *Enterococcus* spp., *Escherichia* spp., *Gastrospirillum* sp., *Helicobacter* spp., *Klebsiella* spp., *Salmonella* spp., *Shigella* spp., *Staphylococcus* spp., *Pseudomonas* spp., *Vibrio* spp., *Yersinia* spp., and the like. Infections that can be treated by the

20   antibodies of the invention include those associated with staph infections (*Staphylococcus aureus*), typhus (*Salmonella typhi*), food poisoning *(Escherichia coli*, such as O157:H7), bascillary dysentery (*Shigella dysenteria*), pneumonia (*Psuedomonas aerugenosa* and/or *Pseudomonas cepacia*), cholera (*Vivrio cholerae*), ulcers (*Helicobacter pylori*), *Salmonella typhimurium* and others. *E. coli*

25   serotype 0157:H7 has been implicated in the pathogenesis of diarrhea, hemorrhagic colitis, hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). The antibodies of the invention are also active against drug-resistant and multiply-drug resistant strains of bacteria, for example, multiply-resistant strains of *Staphylococcus aureus* and vancomycin-resistant strains of *Enterococcus*

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*faecium* and *Enterococcus faecalis*.

In some embodiments, the anti-microbial compositions of the invention are used against gram negative bacteria. In further embodiments, the anti-microbial compositions can be used against *Escherichia* spp., *Pseudomonas* spp., and/or

5 *Salmonella* spp. For example, the anti-microbial compositions can be used against different types of *Escherichia coli, Salmonella typhimurium, Psuedomonas aerugenosa* and other related bacteria.

The anti-microbial compositions of the invention are also effective against viruses. The term "virus" refers to DNA and RNA viruses, viroids, and prions.

10 Viruses include both enveloped and non-enveloped viruses, for example, hepatitis A virus, hepatitis B virus, hepatitis C virus, human immunodeficiency virus (HIV), poxviruses, herpes viruses, adenoviruses, papovaviruses, parvoviruses, reoviruses, orbiviruses, picornaviruses, rotaviruses, alphaviruses, rubivirues, influenza virus type A and B, flaviviruses, coronaviruses, paramyxoviruses, morbilliviruses,

15 pneumoviruses, rhabdoviruses, lyssaviruses, orthmyxoviruses, bunyaviruses, phleboviruses, nairoviruses, hepadnaviruses, arenaviruses, retroviruses, enteroviruses, rhinoviruses and the filovirus.

Other therapeutic conditions that would benefit from the creation or enhancement of reactive oxygen species in a cell, tissue, organ or extracellular

20 compartment are known by those of ordinary skill in the art. For example, such conditions are further described in McCord, Am. J. Med., 108:652-659 (2000), the disclosure of which are hereby incorporated by reference.

Anti-microbial activity can be evaluated against these varieties of microbes using methods available to one of skill in the art. Anti-microbial activity, for

25 example, is determined by identifying the minimum inhibitory concentration (MIC) of an antibody of the present invention that prevents growth of a particular microbial species. In one embodiment, anti-microbial activity is the amount of antibody that kills 50% of the microbes when measured using standard dose or dose response methods.

Methods of evaluating therapeutically effective dosages for treating a microbial infection with antibodies described herein include determining the minimum inhibitory concentration of an antibody preparation at which substantially no microbes grow *in vitro*. Such a method permits calculation of the approximate amount of antibody needed per volume to inhibit microbial growth or to kill 50% of the microbes. Such amounts can be determined, for example, by standard microdilution methods. For example, a series of microbial culture tubes containing the same volume of medium and the substantially the same amount of microbes are prepared, and an aliquot of antibody is added. The aliquot contains differing amounts of antibody in the same volume of solution. The microbes are cultured for a period of time corresponding to one to ten generations and the number of microbes in the culture medium is determined.

The optical density of the cultural medium can also be used to estimate whether microbial growth has occurred – if no significant increase in optical density has occurred, then no significant microbial growth has occurred. However, if the optical density increases, then microbial growth has occurred. To determine how many microbial cells remain alive after exposure to the antibody, a small aliquot of the culture medium can be removed at the time when the antibody is added (time zero) and then at regular intervals thereafter. The aliquot of culture medium is spread onto a microbial culture plate, the plate is incubated under conditions conducive to microbial growth and, when colonies appear, the number of those colonies is counted.

## B. Inhibiting Unwanted Oxidation

The invention also provides methods for ameliorating the negative effects of antibody-mediated production of reactive oxygen species.

The use of molecules that effect the antibody-mediated production of reactive oxygen species is applicable to any situation in which unwanted, deleterious, damaging production of reactive oxidant species that are generated by

antibodies. The molecules that are useful in these situations are referred to generally as "antioxidants," defined as any molecule that has an antagonist effect to an oxidant. An antioxidant so defined includes 1) inhibitors of antibody-mediated superoxide generation, 2) inhibitors of antibody-mediated hydrogen peroxide

5 generation, 3) inhibitors of antibody-mediated ozone generation, 4) inhibitors of the reactions converting hydrogen peroxide into derivative reactive oxidants; and 5) inhibitors of the reactive oxygen species themselves.

Such antioxidants include those that inhibit the activation of oxygen producing reactive oxidants as well as those neutralizing those already formed.

10 The antioxidant effect can occur by any mechanism, including catalysis. Antioxidants as a category include oxygen scavengers or free radical scavengers. Antioxidants may be of different types so they are available if and when they are needed. In view of the presence of oxygen throughout an aerobic organism, antioxidants may be available in different cellular, tissue, organ and extracellular

15 compartments. The latter include extracellular fluid spaces, intraocular fluids, synovial fluid, cerebrospinal fluid, gastrointestinal secretions, interstitial fluid, blood and lymphatic fluid. Antioxidants are present within an organism but are also provided by supplementing the diet and by use of the methods of this invention. In some embodiments, the antioxidants employed include but are not

20 limited to ascorbic acid, α-tocopherol, γ-glutamylcysteinylglycine, γ-glutamyl transpeptidase, α-lipoic acid, dihydrolipoate, Bacetyl-5-methoxytryptamine, flavones, flavonenes, flavanols, catalase, peroxidase, superoxide dismutase, metallothionein, and butylated hydroxytoluene. In other embodiments, the molecule that has the capacity to function as an antioxidant in the context of the

25 methods of this invention is an engineered antibody in which the ability to generate superoxide free radical from reducing singlet oxygen is diminished or preferably absent altogether. Such antibody molecules are described herein.

The use of antioxidants is directed to situations in which an antioxidant is required to prevent, control, minimize, reduce, or inhibit the damage of an oxidant

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or a reactive oxygen product. Thus, the invention contemplates the use of an antioxidant for reducing the antibody-mediated production of reactive oxygen species in tissues, for example, in healthy tissues surrounding the site treated with antibodies. In such situations, without intervention, the cellular damage may result,

5    for example, in inflammatory conditions, in trauma conditions, in organ transplantation and the like.

In the context of using an engineered antibody as an antioxidant, the antibody, having diminished or substantially no ability to generate reactive oxygen species such as superoxide, ozone or hydrogen peroxide since it lacks the reductive

10    centers that reduce singlet oxygen, provides a therapeutic benefit in promoting a desired immune response without inducing additional tissue damage resulting from excess superoxide production. Engineered therapeutic antibody compositions can retain their antigen-binding site so that targeting to a particular antigen is achieved in concert with the desired therapeutic benefits.

15    The present invention further contemplates a method of ameliorating oxidative stress in a subject as well as alleviating a symptom in a subject where the symptom is associated with production of oxidant. Exemplary of conditions in which the therapeutic methods of inhibiting the antibody mediated production of reactive oxygen species with an antioxidant of the present invention include but are

20    not limited to inhibiting aberrant smooth muscle disorder, inhibiting liver disease, proliferation of cancer cells, inhibiting inflammation in cancer patients receiving radiotherapy, inflammatory diseases (arthritis, vasculitis, glomerulonephritis, systemic lupus erythematosus, and adult respiratory distress syndrome), ischemic diseases (heart disease, stroke, intestinal ischemia, and reperfusion injury),

25    hemochromatosis, acquired immunodeficiency syndrome, emphysema, organ transplantation, gastric ulcers, hypertension, preeclampsia, neurological diseases (multiple sclerosis, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and muscular dystrophy) alcoholism and smoking-related diseases.

Cells in which oxidative stress is deleterious include but are not limited to

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endothelial, interstitial, epithelial, muscle (smooth, skeletal or cardiac), phagocytic (including neutrophils and macrophages), white blood cells, dendritic, connective tissue and nervous system cells. Effected tissues include but are not limited to muscle, nervous, skin, glandular, mesenchymal, splenic, sclerous, epithelial and

5    endothelial tissues.

Useful references that describe the use of antioxidants and oxygen scavengers to treat various conditions induced by oxidative stress, other than that relating to the generation of oxidants by an antibody as described in the present invention, include the disclosures of U.S. Patents 5,362,492; 5,599,712; 5,637,315;

10    5,647,315; 5,747,026; 5,848,290; 5,994,339; 6,030,611 and 6,040,611, the disclosures of which patents are hereby incorporated by reference. Such references support the therapeutic uses of antioxidants in the present invention.

**Antibodies**

15    The invention provides therapeutic antibodies. All antibody molecules belong to a family of plasma proteins called immunoglobulins. Their basic building block, the immunoglobulin fold or domain, is used in various forms in many molecules of the immune system and other biological recognition systems. A typical immunoglobulin has four polypeptide chains, contains an antigen binding

20    region known as a variable region, and contains a non-varying region known as the constant region. An antibody contemplated for use in the present invention can be in any of a variety of forms, including a whole immunoglobulin, Fv, Fab, F(ab')$_2$ other fragments, and a single chain antibody that includes the variable domain complementarity determining regions (CDR), or other forms. All of these terms fall

25    under the broad term "antibody" as used herein. The present invention contemplates the use of any specificity of an antibody, polyclonal or monoclonal, and is not limited to antibodies that recognize and immunoreact with a specific antigen. In preferred embodiments, in the context of both the therapeutic and screening methods described herein, an antibody or fragment thereof is used that is

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immunospecific for an antigen.

The term "antibody" as used in this invention includes intact molecules as well as fragments thereof, such as Fab, F(ab')$_2$, and Fv, which are capable of binding an epitope. These antibody fragments retain some ability to selectively bind with its antigen or receptor and are defined as follows:

(1)    Fab, the fragment, which contains a monovalent antigen-binding fragment of an antibody molecule, can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain;

(2)    Fab', the fragment of an antibody molecule can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule;

(3)    F(ab')$_2$, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; F(ab')$_2$ is a dimer of two Fab' fragments held together by two disulfide bonds;

(4)    Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and

(5)    Single chain antibody ("sFv"), defined as a genetically engineered molecule containing the variable region of the light chain, the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

The preparation of polyclonal antibodies is well-known to those skilled in the art. See, for example, Green, et al., Production of Polyclonal Antisera, in: Immunochemical Protocols (Manson, ed.), pages 1-5 (Humana Press); Coligan, et al., Production of Polyclonal Antisera in Rabbits, Rats Mice and Hamsters, in: Current Protocols in Immunology, section 2.4.1 (1992), which are hereby incorporated by reference.

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The preparation of monoclonal antibodies is also conventional. See, for example, Kohler & Milstein, Nature, 256:495 (1975); Coligan, et al., sections 2.5.1-2.6.7; and Harlow, et al., in: Antibodies: A Laboratory Manual, page 726 (Cold Spring Harbor Pub. (1988)), which are hereby incorporated by reference.

5 Monoclonal antibodies can be isolated and purified from hybridoma cultures by a variety of well-established techniques. Such isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography. See, e.g., Coligan, et al., sections 2.7.1-2.7.12 and sections 2.9.1-2.9.3; Barnes, et al., Purification of Immunoglobulin G (IgG), in:

10 Methods in Molecular Biology, Vol. 10, pages 79-104 (Humana Press (1992).

The present invention also contemplates engineered therapeutic molecules including engineered antibodies that have been altered to contain an additional reductive center. Such engineered antibody molecules can be used where an insufficient amount of antibody or reactive oxygen species is present. Such

15 engineered therapeutic molecules can be engineered to have an increased number of reductive centers relative to those that were naturally occurring in the molecule or antibody.

Introduction of a reductive center in a engineered molecule or antibody is accomplished by methods well known to one of ordinary skill in the art. Preferred

20 means including recombinant expression methods and well as direct protein synthesis methods have been previously described. The choice of method is necessarily dependent on the length of the molecule being engineered. Regardless of the methods employed, the positioning, i.e., the location, of the engineered reductive center is based upon the ability of the engineered molecule to exhibit

25 reducing activity on singlet oxygen. Preferably, the incorporated reductive centers are positioned such that they are deeply buried in the folded molecule and so reactive oxygen species production is retained or augmented. In one embodiment, an engineered antibody retains antigen-binding function, and the location of an engineered reductive center is adjacent to a variable binding domain. In certain

30 aspects, one reductive center is contemplated. In other aspects, two reductive centers are contemplated. Still, in other aspects, more than three reductive centers

are contemplated. Preferably, the reductive centers comprise indole. Also

contemplated are reductive centers having indole moieties such as those present in

tryptophan residue. Any technique to engineer such reductive centers in a molecule

or antibody is contemplated for use in the present invention. In a preferred

5      embodiment, the reductive centers are introduced by site-directed mutagenesis of

nucleotide sequences encoding the engineered antibody such that the substituted

nucleotides encode tryptophan residues at predetermined locations in the encoded

molecule.

In the embodiment of preparing an engineered molecule such as an antibody

10     to include desired reductive centers, a molecule that is produced by recombinant

technology is also contemplated to be in the form of a fusion conjugate, where the

conjugate can provide a sensitizer molecule as described herein for use in

therapeutic methods as described herein.

Engineered antibodies or other molecules, which can be any protein or

15     polypeptide that contains reductive centers that function according to the methods

of the invention and/or sensitizer molecules, are contemplated for any of the

methods as described herein.

Methods of *in vitro* and *in vivo* manipulation of monoclonal antibodies are

well known to those skilled in the art. One particular manipulation involves the

20     process of humanizing a monoclonal antibody by recombinant means to generate

antibodies containing human specific and recognizable sequences. See, for review,

Holmes, et al., J. Immunol., 158:2192-2201 (1997) and Vaswani, et al., Annals

Allergy, Asthma & Immunol., 81:105-115 (1998).

Methods of making antibody fragments are known in the art (see for

25     example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring

Harbor Laboratory, New York, (1988), incorporated herein by reference).

Antibody fragments of the present invention can be prepared by proteolytic

hydrolysis of the antibody or by expression in *E. coli* of DNA encoding the

fragment. Antibody fragments can be obtained by pepsin or papain digestion of

whole antibodies conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted F(ab')$_2$. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting

5    from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly. These methods are described, for example, in U.S. Patents No. 4,036,945 and No. 4,331,647, and references contained therein. These patents are hereby incorporated in their entireties by reference.

10    Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody. For example, Fv fragments comprise an association of $V_H$ and $V_L$ chains. This

15    association may be noncovalent or the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde. Preferably, the Fv fragments comprise $V_H$ and $V_L$ chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the $V_H$ and $V_L$

20    domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example, by Whitlow, et al., Methods: a Companion to Methods in

25    Enzymology, Vol. 2, page 97 (1991); Bird, et al., Science, 242:423-426 (1988); Ladner, et al, US Patent No. 4,946,778; and Pack, et al., Bio/Technology, 11:1271-77 (1993).

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition

units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells. See, for example, Larrick, et al., Methods: a Companion to Methods in Enzymology, Vol. 2, page 106 (1991).

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The invention contemplates human and humanized forms of non-human (e.g. murine) antibodies. Such humanized antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')$_2$ or other antigen-binding subsequences of antibodies) that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibodies) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a nonhuman species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity.

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In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, humanized antibodies will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see: Jones et al., Nature 321, 522-525 (1986); Reichmann et al., Nature 332, 323-329 (1988); Presta, Curr. Op. Struct. Biol. 2, 593-596 (1992); Holmes, et al., J. Immunol., 158:2192-2201 (1997) and Vaswani, et al., Annals Allergy, Asthma & Immunol., 81:105-115 (1998).

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The invention also provides methods of mutating antibodies to optimize their affinity, selectivity, binding strength, reactive oxygen species production or other desirable property. A mutant antibody refers to an amino acid sequence variant of an antibody. In general, one or more of the amino acid residues in the mutant

5      antibody is different from what is present in the reference antibody. Such mutant antibodies necessarily have less than 100% sequence identity or similarity with the reference amino acid sequence. In general, mutant antibodies have at least 75% amino acid sequence identity or similarity with the amino acid sequence of either the heavy or light chain variable domain of the reference antibody. Preferably,

10     mutant antibodies have at least 80%, more preferably at least 85%, even more preferably at least 90%, and most preferably at least 95% amino acid sequence identity or similarity with the amino acid sequence of either the heavy or light chain variable domain of the reference antibody.

One method of mutating antibodies involves affinity maturation using phage

15     display. Affinity maturation using phage display refers to a process described in Lowman et al., Biochemistry 30(45): 10832-10838 (1991), see also Hawkins et al., J. Mol Biol. 254: 889-896 (1992). While not strictly limited to the following description, this process can be described briefly as involving mutation of several antibody hypervariable regions in a number of different sites with the goal of

20     generating all possible amino acid substitutions at each site. The antibody mutants thus generated are displayed in a monovalent fashion from filamentous phage particles as fusion proteins. Fusions are generally made to the gene III product of M13. The phage expressing the various mutants can be cycled through several rounds of selection for the trait of interest, e.g. binding affinity or selectivity. The

25     mutants of interest are isolated and sequenced. Such methods are described in more detail in U.S. Patent 5,750,373, U.S. Patent 6,290,957 and Cunningham, B. C. et al., EMBO J. 13(11), 2508-2515 (1994).

The preparation of a therapeutic antibody (or fragment thereof) of this invention can be accomplished by recombinant expression techniques as well as

protein synthesis, methods of which are well known to one of ordinary skill in the art. For recombinant approaches, mutation of a nucleic acid that encodes an antibody or fragment thereof can be conducted by a variety of means, but is most conveniently conducted using mutagenized oligonucleotides that are designed to

5    introduce mutations at predetermined sites that then encode an altered amino acid sequence in the expressed molecule. Such alterations include substitutions, additions, and/or deletions of particular nucleotide sequences that similarly encode substitutions, additions, and/or deletions of the encoded amino acid residue sequence. Site-directed mutagenesis, also referred to as oligonucleotide-directed

10   mutagenesis and variations thereof, and the subsequent cloning of the altered genes are well known techniques (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Chapter 15, Cold Spring Harbor Laboratory Press, (1989)). Another recombinant approach includes synthesizing the gene encoding a therapeutic molecule of this invention by combining long oligonucleotide strands

15   that are subsequently annealed and converted to double-stranded DNA suitable for cloning and expression (Ausebel et al., Current Protocols in Molecular Biology, Units 10 and 15, Wiley and Sons, Inc. (2000)). Such techniques can be used to create engineered molecules that contain a reduction center and are able to generate hydrogen peroxide or superoxide from singlet oxygen. It is contemplated that such

20   engineered molecules can be designed based on antibody structure and on the T-cell receptor, in the case of hydrogen peroxide.

Thus, the present invention contemplates an antibody that has been engineered to generate more superoxide free radical, ozone or hydrogen peroxide in a desired location. The antibody is engineered to contain additional reductive

25   centers that increase the reduction of singlet molecular oxygen to superoxide free radical or hydrogen peroxide, as described in examples I and II herein. The invention also contemplates an antibody that has been engineered to have at least a diminished capacity to generate superoxide free radical or hydrogen peroxide from singlet oxygen. In that context, the antibody lacks at least one of its reductive

centers and preferably is substantially free of a reductive center. Such antibody compositions are readily prepared with methods well known to one of ordinary skill in the art.

If desired, polyclonal or monoclonal antibodies prepared for use as
5    therapeutic compositions or in the methods of invention can be further purified, for example, by binding to and elution from a matrix to which the polypeptide or a peptide to which the antibodies were raised is bound. Those of skill in the art will know of various techniques common in the immunology arts for purification and/or concentration of polyclonal antibodies, as well as monoclonal antibodies (Coligan,
10   et al., Unit 9, Current Protocols in Immunology, Wiley Interscience, (1991)).


**Compositions**

The antibodies, antioxidants and oxygen scavengers of the invention may be formulated into a variety of acceptable compositions. Such pharmaceutical
15   compositions can be administered to a mammalian host, such as a human patient, in a variety of forms adapted to the chosen route of administration, *i.e.*, orally or parenterally, by intravenous, intramuscular, topical or subcutaneous routes.

In cases where compounds, for example, antioxidant and oxygen scavenger compounds, are sufficiently basic or acidic to form stable nontoxic acid or base
20   salts, administration of such compounds as salts may be appropriate. Examples of pharmaceutically acceptable salts are organic acid addition salts formed with acids that form a physiological acceptable anion, for example, tosylate, methanesulfonate, acetate, citrate, malonate, tartarate, succinate, benzoate, ascorbate, α-ketoglutarate, and α-glycerophosphate. Suitable inorganic salts may
25   also be formed, including hydrochloride, sulfate, nitrate, bicarbonate, and carbonate salts.

Pharmaceutically acceptable salts are obtained using standard procedures well known in the art, for example by reacting a sufficiently basic compound such as an amine with a suitable acid affording a physiologically acceptable anion.

Alkali metal (for example, sodium, potassium or lithium) or alkaline earth metal (for example calcium) salts of carboxylic acids also are made.

Thus, the present antibodies and compounds may be systemically administered, *e.g.*, orally, in combination with a pharmaceutically acceptable vehicle such as an inert diluent or an assimilable edible carrier. They may be enclosed in hard or soft shell gelatin capsules, may be compressed into tablets, or may be incorporated directly with the food of the patient's diet. For oral therapeutic administration, the antibodies, antioxidants and oxygen scavengers may be combined with one or more excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60% of the weight of a given unit dosage form. The amount of antibodies, antioxidants or oxygen scavengers in such therapeutically useful compositions is such that an effective dosage level will be obtained.

The tablets, troches, pills, capsules, and the like may also contain the following: binders such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, fructose, lactose or aspartame or a flavoring agent such as peppermint, oil of wintergreen, or cherry flavoring may be added. When the unit dosage form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier, such as a vegetable oil or a polyethylene glycol. Various other materials may be present as coatings or to otherwise modify the physical form of the solid unit dosage form. For instance, tablets, pills, or capsules may be coated with gelatin, wax, shellac or sugar and the like. A syrup or elixir may contain the active compound, sucrose or fructose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or

orange flavor. Of course, any material used in preparing any unit dosage form should be pharmaceutically acceptable and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and devices.

For wound healing, topical application to a wound on a subject can be employed. A composition containing an antibody can be applied directly to the wound or applied to a bandage and then applied to the wound.

The active compound may also be administered intravenously or intraperitoneally by infusion or injection. Solutions of the active compound or its salts may be prepared in water, optionally mixed with a nontoxic surfactant. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, triacetin, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical dosage forms suitable for injection or infusion can include sterile aqueous solutions or dispersions or sterile powders comprising the active ingredient that are adapted for the extemporaneous preparation of sterile injectable or infusible solutions or dispersions, optionally encapsulated in liposomes. In all cases, the ultimate dosage form should be sterile, fluid and stable under the conditions of manufacture and storage. The liquid carrier or vehicle can be a solvent or liquid dispersion medium comprising, for example, water, ethanol, a polyol (for example, glycerol, propylene glycol, liquid polyethylene glycols, and the like), vegetable oils, nontoxic glyceryl esters, and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the formation of liposomes, by the maintenance of the required particle size in the case of dispersions or by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, buffers or sodium chloride. Prolonged absorption of the injectable compositions can be brought about

by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the antibodies, antioxidants or oxygen scavengers in the required amount in the appropriate solvent

5    with various of the other ingredients enumerated above, as required, followed by filter sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying techniques, which yield a powder of the antibodies, antioxidants or oxygen scavengers plus any additional desired ingredient present in the

10   previously sterile-filtered solutions.

For topical administration, the antibodies, antioxidants or oxygen scavengers may be applied in pure form, i.e., when they are liquids. However, it will generally be desirable to administer them to the skin as compositions or formulations, in combination with a dermatologically acceptable carrier, which

15   may be a solid or a liquid.

Useful solid carriers include finely divided solids such as talc, clay, microcrystalline cellulose, silica, alumina and the like. Useful liquid carriers include water, alcohols or glycols or water-alcohol/glycol blends, in which the present antibodies, antioxidants or oxygen scavengers can be dissolved or dispersed

20   at effective levels, optionally with the aid of non-toxic surfactants. Adjuvants such as fragrances and additional antimicrobial agents can be added to optimize the properties for a given use. The resultant liquid compositions can be applied from absorbent pads, used to impregnate bandages and other dressings, or sprayed onto the affected area using pump-type or aerosol sprayers.

25   Thickeners such as synthetic polymers, fatty acids, fatty acid salts and esters, fatty alcohols, modified celluloses or modified mineral materials can also be employed with liquid carriers to form spreadable pastes, gels, ointments, soaps, and the like, for application directly to the skin of the user.

Examples of useful dermatological compositions that can be used to deliver

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the antibodies, antioxidants or oxygen scavengers of the present invention to the skin are known to the art; for example, see Jacquet et al. (U.S. Pat. No. 4,608,392), Geria (U.S. Pat. No. 4,992,478), Smith et al. (U.S. Pat. No. 4,559,157) and Wortzman (U.S. Pat. No. 4,820,508).

5       Useful dosages of the antibodies, antioxidants or oxygen scavengers of the present invention can be determined by comparing their *in vitro* activity, and *in vivo* activity in animal models. Methods for the extrapolation of effective dosages in mice, and other animals, to humans are known to the art; for example, see U.S. Pat. No. 4,938,949.

10       Generally, the concentration of the antibodies, antioxidants or oxygen scavengers of the present invention in a liquid composition, such as a lotion, will be from about 0.1-25 wt-%, preferably from about 0.5-10 wt-%. The concentration in a semi-solid or solid composition such as a gel or a powder will be about 0.1-5 wt-%, preferably about 0.5-2.5 wt-%.

15       The amount of the antibodies, antioxidants or oxygen scavengers, or an active salt or derivative thereof, required for use in treatment will vary not only with the particular salt selected but also with the route of administration, the nature of the condition being treated and the age and condition of the patient and will be ultimately at the discretion of the attendant physician or clinician.

20       In general, however, a suitable dose will be in the range of from about 0.5 to about 100 mg/kg, e.g., from about 10 to about 75 mg/kg of body weight per day, such as 3 to about 50 mg per kilogram body weight of the recipient per day, preferably in the range of 6 to 90 mg/kg/day, most preferably in the range of 15 to 60 mg/kg/day.

25       The antibodies, antioxidants or oxygen scavengers are conveniently administered in unit dosage form; for example, containing 5 to 1000 mg, conveniently 10 to 750 mg, most conveniently, 50 to 500 mg of active ingredient per unit dosage form.

Ideally, the antibodies, antioxidants or oxygen scavengers should be

administered to achieve peak plasma concentrations of the active compound of from about 0.5 to about 75 µM, preferably, about 1 to 50 µM, most preferably, about 2 to about 30 µM. This may be achieved, for example, by the intravenous injection of a 0.05 to 5% solution of the antibodies, antioxidants or oxygen

5       scavengers, optionally in saline, or orally administered as a bolus containing about 1-100 mg of the antibodies, antioxidants or oxygen scavengers. Desirable blood levels may be maintained by continuous infusion to provide about 0.01-5.0 mg/kg/hr or by intermittent infusions containing about 0.4-15 mg/kg of the antibodies, antioxidants or oxygen scavengers.

10      The desired dose may conveniently be presented in a single dose or as divided doses administered at appropriate intervals, for example, as two, three, four or more sub-doses per day. The sub-dose itself may be further divided, e.g., into a number of discrete loosely spaced administrations; such as multiple inhalations from an insufflator or by application of a plurality of drops into the eye.

15      In a preferred embodiment, an antioxidant enters the cell and reacts with the reactive oxygen species thereby reducing the concentration of reactive oxygen species in the cell. In an alternative embodiment, an antioxidant enters the cell or is present in the surrounding extracellular milieu and reacts with the oxidants generated from reactive oxygen species.

20      The therapeutic compositions of this invention, the antioxidants described herein, antibodies that include both engineered antibodies and other molecules containing additional reductive centers as described herein for promoting antibody activity, are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered and timing

25      depends on the subject to be treated, capacity of the subject's system to utilize the active ingredient, and degree of therapeutic effect desired. Precise amounts of active ingredient required to be administered depend on the judgement of the practitioner and are peculiar to each individual. However, suitable dosage ranges for various types of applications depend on the route of administration. Suitable

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regimes for administration are also variable, but are typified by an initial administration followed by repeated doses at intervals to result in the desired outcome of the therapeutic treatment.

Antibodies, antioxidants or oxygen scavengers contemplated for use in the
5    present invention can be delivered to the site of interest to mediate the desired outcome in a composition such as a liposome, the preparation of which is well known to one of ordinary skill in the art of liposome-mediated delivery. Alternative delivery means include but are not limited to administration intravenously, topically, orally, by inhalation, by cannulation, intracavitally,
10    intramuscularly, transdermally, and subcutaneously.

Therapeutic compositions of the present invention contain a physiologically tolerable carrier together with an antioxidant as described herein or an antibody as described herein for providing antibody activity, dissolved or dispersed therein as an active ingredient. In a preferred embodiment, the therapeutic composition is not
15    immunogenic when administered to a mammal or human patient for therapeutic purposes.

The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well understood in the art and need not be limited based on formulation. Typically such compositions are prepared as
20    injectables either as liquid solutions or suspensions, however, solid forms suitable for solution, or suspensions, in liquid prior to use can also be prepared. The preparation can also be emulsified.

The active ingredients can be mixed with excipients that are pharmaceutically acceptable and compatible with the active ingredient and in
25    amounts suitable for use in the therapeutic methods described herein. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like which enhance the effectiveness of the active

ingredient.

The therapeutic compositions of the present invention can include pharmaceutically acceptable salts of the components therein. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups

5    of the polypeptide) that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-

10   ethylamino ethanol, histidine, procaine and the like.

Physiologically tolerable carriers are well known in the art. Exemplary of liquid carriers are sterile aqueous solutions that contain no materials in addition to the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH value, physiological saline or both, such as phosphate-buffered

15   saline. Still further, aqueous carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, polyethylene glycol and other solutes.

Liquid compositions can also contain liquid phases in addition to and to the exclusion of water. Exemplary of such additional liquid phases are glycerin,

20   vegetable oils such as cottonseed oil, and water-oil emulsions.

Other therapeutic conditions that would benefit from the antioxidant inhibition of antibody mediated oxidant production in a cell, tissue, or organs as well as extracellular compartments are well known to those of ordinary skill in the art and have been reviewed by McCord, Am. J. Med., 108:652-659 (2000) and

25   Babior et al., Am. J. Med., 109:33-44 (2000), the disclosures of which are hereby incorporated by reference.

The invention is further described in detail by reference to the non-limiting examples that follow. While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that

modifications and variations are within the spirit and scope of that which is
described and claimed.

## Example I: Antibodies have an intrinsic capacity to destroy antigens

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## Materials and Methods

Antibodies: The following whole antibodies were obtained from
PharMingen: 49.2 (mouse $IgG_{2b}$ κ), G155-178 (mouse $IgG_{2a}$ κ), 107.3 (mouse $IgG_1$
κ), A95-1 (rat $IgG_{2b}$), G235-2356 (hamster IgG), R3-34 (rat IgG κ), R35-95 (rat
$IgG_{2a}$ κ), 27-74 (mouse IgE), A110-1 (rat $IgG_1$ λ), 145-2C11 (hamster IgG group1

10 κ), M18-254 (mouse IgA κ), and MOPC-315 (mouse IgA λ). The following were
obtained from Pierce: 31243 (sheep IgG), 31154 (human IgG), 31127 (horse IgG),
and 31146 (human IgM).

The following $F(ab')_2$ fragments were obtained from Pierce: 31129 (rabbit
IgG), 31189 (rabbit IgG), 31214 (goat IgG), 31165 (goat IgG), and 31203 (mouse

15 IgG). Protein A, protein G, trypsin-chymotrypsin inhibitor (Bowman-Birk
inhibitor), β-lactoglobulin A, α-lactalbumin, myoglobin, β-galactosidase, chicken
egg albumin, aprotinin, trypsinogen, lectin (peanut), lectin (Jacalin), BSA,
superoxide dismutase, and catalase were obtained from Sigma. Ribonuclease I A
was obtained from Amersham Pharmacia. The following immunoglobulins were

20 obtained in-house using hybridoma technology: OB2-34C12 (mouse $IgG_1$ κ),
SHO1-41G9 (mouse $IgG_1$ κ), OB3-14F1 (mouse $IgG_{2a}$ κ), DMP-15G12 (mouse
$IgG_{2a}$ κ), AD1-19G1 (mouse $IgG_{2b}$ κ), NTJ-92C12 (mouse $IgG_1$ κ), NBA-5G9
(mouse $IgG_1$ κ), SPF-12H8 (mouse $IgG_{2a}$ κ), TIN-6C11 (mouse $IgG_{2a}$ κ), PRX-1B7
(mouse $IgG_{2a}$ κ), HA5-19A11 (mouse $IgG_{2a}$ κ), EP2-19G2 (mouse $IgG_1$ κ), GNC-

25 92H2 (mouse $IgG_1$ κ), WD1-6G6 (mouse $IgG_1$ κ), CH2-5H7 (mouse $IgG_{2b}$ κ), PCP-
21H3 (mouse $IgG_1$ κ), and TM1-87D7 (mouse $IgG_1$ κ). DRB polyclonal (human
IgG) and DRB-b12 (human IgG) were supplied by Dennis R. Burton (The Scripps
Research Institute). 1D4 Fab (crystallized) was supplied by Ian A. Wilson (The
Scripps Research Institute).

All assays were carried out in PBS (10 mM phosphate/160 mM sodium chloride, pH 7.4). Commercial protein solution samples were dialyzed into PBS as necessary. Amplex Red hydrogen peroxide assay kits (A-12212) were obtained from Molecular Probes.

5          Antibody/Protein Irradiation. Unless otherwise stated, the assay solution (100 μl, 6.7 μM protein in PBS, pH 7.4) was added to a glass vial, sealed with a screw-cap, and irradiated with either UV (312 nm, 8000 μWcm$^{-2}$ Fischer-Biotech transilluminator) or visible light.

Quantitative Assay for Hydrogen Peroxide. An aliquot (20 μl) from the

10          protein solution was removed and added into a well of a 96-well microtiter plate (Costar) containing reaction buffer (80 μl). Working solution (100 μl/400 μM Amplex Red reagent 1/2 units/ml horseradish peroxidase) was then added, and the plate was incubated in the dark for 30 min. The fluorescence of the well components was then measured using a CytoFluor Multiwell Plate Reader (Series

15          4000, PerSeptive Biosystems, Framingham, MA; Ex/Em: 530/580 nm). The hydrogen peroxide concentration was determined using a standard curve. All experiments were run in duplicate, and the rate is quoted as the mean of at least two measurements.

Sensitization and Quenching Assays. A solution of 31127 (100 μl of horse

20          IgG, 6.7 μM) in PBS (pH 7.4, 4% dimethylformamide) and hematoporphyrin IX (40 μM) was placed in proximity to a strip light. Hydrogen peroxide concentration was determined as described herein. The assay was also performed in the presence of NaN$_3$ (100 mM) or PBS in D$_2$O.

Oxygen Dependence. A solution of 31127 (1.6 ml, horse IgG, 6.7 μM) in

25          PBS (pH 7.4) was rigorously degassed using the freeze/thaw method under argon. Aliquots (100 μl) were introduced into septum-sealed glass vials that had been purged with the appropriate O$_2$/Ar mixtures (0-100%) via syringe. Dissolved oxygen concentrations were measured with an Orion 862A dissolved oxygen meter. These solutions were then vortexed vigorously, allowed to stand for 20 min, and

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then vortexed again. A syringe containing the requisite $O_2$/Ar mixture was used to maintain atmospheric pressure during the course of the experiment. Aliquots (20 µl) were removed using a gas-tight syringe and hydrogen peroxide concentration measured as described herein. The data from three separate experiments were

5    collated and analyzed using the Enzyme Kinetics v1.1 computer program (for determination of $V_{max}$ and $K_m$ parameters).

       Antibody Production of Hydrogen Peroxide in the Dark, Using a Chemical $^1O_2$ Source. A solution of sheep IgG 31243 (100 µl, 20 µM) in PBS (pH 7.4) and the endoperoxide of disodium 3,3N-(1,4-naphthylidene) dipropionate (25 mM in

10    $D_2O$) were placed in a warm room (37EC) for 30 min in the dark. Hydrogen peroxide concentration was determined as described herein.

       Hydrogen Peroxide Formation by the Fab1D4 Crystal. A suspension of crystals of the Fab fragment of 1D4 (2 µl) was diluted with PBS (198 µl, pH 7.4) and vortexed gently. Following centrifugation, the supernatant was removed, and

15    the washing procedure was repeated twice further. The residual crystal suspension was then diluted into PBS, pH 7.4 (100 µl), and added into a well of a quartz ELISA plate. Following UV irradiation for 30 min, Amplex Red working solution (100 µl) was added, and the mixture was viewed on a fluorescence microscope.

       Antibody Fluorescence Versus Hydrogen Peroxide Formation. A solution

20    of 31127 (1.0 ml of horse IgG, 6.7 µM) in PBS (pH 7.4) was placed in a quartz cuvette and irradiated with UV light for 40 min. At 10-min intervals, the fluorescence of the solution was measured using an SPF-500C spectrofluorimeter (SLM-Aminco, Urbana, IL; Ex/Em, 280/320). At the same time point, an aliquot (20 µl) of the solution was removed, and the hydrogen peroxide concentration was

25    determined as described herein.

       Consumption of Hydrogen Peroxide by Catalase. A solution of EP2-19G12 (100 µl of mouse IgG, 20 µM in PBS, pH 7.4) was irradiated with UV light for 30 min, after which time the concentration of hydrogen peroxide was determined by stick test (EM Quant Peroxide Test Sticks) to be 2 mg/liter. Catalase [1 µl, Sigma,

3. 2 M $(NH_4)_2SO_4$, pH 6.0] was added, and after 1 min, the concentration of $H_2O_2$ was found to be 0 mg/liter.

Denaturation. IgG 19G12 (100 µl, 6.7 µM) was heated to 100EC in an Eppendorf tube for 2 min. The resultant solution was transferred to a glass, screw-cap vial and irradiated with UV light for 30 min. The concentration of $H_2O_2$ was determined after 30 min.

## Results and Discussion

The measured values for the initial rate of formation of hydrogen peroxide by a panel of intact immunoglobulins and antibody fragments are collected in Table 1. It is believed that Ig-generated $O_2^{\bullet-}$ dismutates spontaneously into $H_2O_2$, which is then utilized as a cosubstrate with $N$-acetyl-3,7-dihydroxyphenazine 1 (Amplex Red) for horseradish peroxidase, to produce the highly fluorescent resorufin 2 (excitation maxima 563 nm, emission maxima 587 nm) (FIG. 2) (Zhou, M., Diwu, Z., Panchuk-Voloshina, N. & Haugland, R. P., Anal. Biochem., 253, 162-168 (1997)). To confirm that irradiation of the buffer does not generate $O_2^{\bullet-}$ and that the antibodies are not simply acting as protein dismutases (Petyaev, I. M. & Hunt, J. V., Redox Report, 2, 365-372 (1996)), the enzyme superoxide dismutase was irradiated in PBS. Under these conditions, the rate of hydrogen peroxide generation is the same as irradiation of PBS alone.

### Table 1. Production of hydrogen peroxide* by immunoglobulins

| Entry | Clone | Source | Isotype | Rate,[H] nmol/min/mg |
|---|---|---|---|---|
| 1 | CH25H7 | Mouse | IgG2b, κ | 0.25 |
| 2 | WD16G6 | Mouse | IgG1, κ | 0.24 |
| 3 | SHO-141G9 | Mouse | IgG1, κ | 0.26 |
| 4 | OB234C12 | Mouse | IgG1, κ | 0.22 |
| 5 | OB314F1 | Mouse | IgG2a, κ | 0.23 |
| 6 | DMP15G12 | Mouse | IgG2a, κ | 0.18 |
| 7 | AD19G1 | Mouse | IgG2b, κ | 0.22 |

| Entry | Clone | Source | Isotype | Rate,[H] nmol/min/mg |
|-------|-------|--------|---------|-------------------|
| 8 | NTJ92C12 | Mouse | IgG1, κ | 0.17 |
| 9 | NBA5G9 | Mouse | IgG1, κ | 0.17 |
| 10 | SPF12H8 | Mouse | IgG2a, κ | 0.29 |
| 11 | TIN6C11 | Mouse | IgG2a, κ | 0.24 |
| 12 | PRX1B7 | Mouse | IgG2a, κ | 0.22 |
| 13 | HA519A4 | Mouse | IgG1, κ | 0.20 |
| 14 | 92H2 | Mouse | IgG1, κ | 0.41 |
| 15 | 19G2 | Mouse | IgG1, κ | 0.20 |
| 16 | PCP-21H3 | Mouse | IgG1, κ | 0.97 |
| 17 | TM1-87D7 | Mouse | IgG1, κ | 0.28 |
| 18 | 49.2 | Mouse | IgG2b, κ | 0.24 |
| 19 | 27-74 | Mouse | IgE, std. isotype | 0.36 |
| 20 | M18-254 | Mouse | IgA, κ | 0.39 |
| 21 | MOPC-315 | Mouse | IgA, λ | 0.39 |
| 22 | 31203 | Mouse | F(ab')$_2$ | 0.21 |
| 23 | b12 | Human | IgG | 0.45 |
| 24 | polyclonal | Human | IgG | 0.34 |
| 25 | 31154 | Human | IgG | 0.18 |
| 26 | 31146 | Human | IgM | 0.22 |
| 27 | R3-34 | Rat | IgG1, κ | 0.27 |
| 28 | R35-95 | Rat | IgG2a, κ | 0.17 |
| 29 | A95-1 | Rat | IgG2b | 0.15 |
| 30 | A110-1 | Rat | IgG1, λ | 0.34 |
| 31 | G235-2356 | Hamster | IgG | 0.24 |
| 32 | 145-2C11 | Hamster | IgG, gp 1, κ | 0.27 |
| 33 | 31243 | Sheep | IgG | 0.20 |
| 34 | 31127 | Horse | IgG | 0.18 |
| 35 | polyclonal | Horse | IgG | 0.34 |
| 36 | 31229 | Rabbit | F(ab')$_2$ | 0.19 |
| 37 | 31189 | Rabbit | F(ab')$_2$ | 0.14 |
| 38 | 31214 | Goat | F(ab')$_2$ | 0.24 |

| Entry | Clone | Source | Isotype | Rate,$^H$ nmol/min/mg |
|-------|-------|--------|---------|-----------------------|
| 39 | 31165 | Goat | F(ab')$_2$ | 0.25 |

\* Assay conditions are described in *Materials and Methods*.

$^H$ Mean values of at least two determinations. The background rate of $H_2O_2$ formation is 0.005 nmol/min in PBS and 0.003 nm/min in PBS with SOD.

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The rates of hydrogen peroxide formation were linear for more than 10% of the reaction, with respect to the oxygen concentration in PBS under ambient conditions (275 µM). With sufficient oxygen availability, the antibodies can

10 generate at least 40 equivalents of $H_2O_2$ per protein molecule without either a significant reduction in activity or structural fragmentation. An example of the initial time course of hydrogen peroxide formation in the presence or absence of antibody 19G2 is shown in FIG. 3A. This activity is lost following denaturation of the protein by heating.

15 The data in Table 1 reveal a universal ability of antibodies to generate $H_2O_2$ from $^1O_2$. This function seems to be shared across a range of species and is independent of the heavy and light chain compositions investigated or antigen specificity. The initial rates of hydrogen peroxide formation for the intact antibodies are highly conserved, varying from 0.15 nmol/min/mg [clone A95-1(rat

20 IgG2b)] to 0.97 nmol/min/mg (clone PCP-21H3, a murine monoclonal IgG) across the whole panel. Although the information available is more limited for the component antibody fragments, the activity seems to reside in both the Fab and F(ab')$_2$ fragments.

If this activity were due to a contaminant, it would have to be present in

25 every antibody and antibody fragment obtained from diverse sources. However, to further rule out contamination, crystals of the murine antibody 1D4 Fab from which high-resolution x-ray structures have been obtained (at 1.7 D) were investigated for their ability to generate $H_2O_2$ (FIG. 4). Reduction of $^1O_2$ is clearly observed in these crystals.

Investigations into this antibody transformation support singlet oxygen as the intermediate being reduced. No formation of hydrogen peroxide occurs with antibodies under anaerobic conditions either in the presence or absence of UV irradiation. Furthermore, no generation of hydrogen peroxide occurs under

5    ambient aerobic conditions without irradiation. Irradiation of antibodies with visible light in the presence of a known photosensitizer of $^3O_2$ in aqueous solutions hematoporphyrin (HP) (Kreitner, M., Alth, G., Koren, H., Loew, S. & Ebermann, R., Anal. Biochem., 213, 63-67 (1993)), leads to hydrogen peroxide formation (FIG. 5A). The curving in the observed rates is due to consumption of oxygen

10    from within the assay mixture. Concerns that the interaction between photoexcited HP and oxygen may be resulting in $O_2^{\bullet-}$ formation (Beauchamp, C. & Fridovich, I., Anal. Biochem., 44, 276-287 (1971); Srinivasan, V. S., Podolski, D., Westrick, N. J. & Neckers, D. C., J. Am. Chem. Soc., 100, 6513-6515 (1978)) were largely discounted by suitable background experiments with the sensitizer alone (data

15    shown in FIG. 5A). The efficient formation of $H_2O_2$ with HP and visible light both reaffirm the intermediacy of $^1O_2$ and show that UV radiation is not necessary for the Ig to perform this reduction.

Furthermore, incubation of sheep antibody 31243 in the dark at 37EC, with a chemical source of $^1O_2$ [the endoperoxide of 3N,3N-(1,4-naphthylidene)

20    dipropionate] leads to hydrogen peroxide formation.

The rate of formation of $H_2O_2$, by horse IgG with HP (40 μM) in visible light, is increased in the presence of $D_2O$ and reduced with the $^1O_2$ quencher $NaN_3$ (40 mM) (FIG. 5B) (Hasty, N., Merkel, P. B., Radlick, P. & Kearns, D. R. Tetrahedron Lett., 49-52 (1972)). The substitution of $D_2O$ for $H_2O$ is known to

25    promote $^1O_2$-mediated processes via an increase of approximately 10-fold in its lifetime (Merkel, P. B., Nillson, R. & Kearns, D. R., J. Am. Chem. Soc., 94, 1030-1031 (1972)).

The rate of hydrogen peroxide formation is proportional to IgG concentration between 0.5 and 20 μM but starts to curve at higher concentrations

30    (FIG. 5C). The lifetime of $^1O_2$ in protein solution is expected to be lower than in pure water due to the opportunity for reaction. It is therefore thought that the

observed curvature may be due to a reduction in the lifetime of $^1O_2$ due to reaction with the antibody.

Significantly, the effect of oxygen concentration on the observed rate of $H_2O_2$ production shows a significant saturation about 200 μM of oxygen (FIG. 5D).

5    Therefore, the mechanism of reduction may involve either one or more oxygen binding sites within the antibody molecule. By treating the raw rate data to nonlinear regression analysis and by fitting to the Michaelis-Menten equation, a $K_m$app($O_2$) of 187 μM and a $V_{max}$app of 0.4 nmol/min/mg are obtained. This antibody rate is equivalent to that observed for mitochondrial enzymes that reduce

10   molecular oxygen *in vivo*.

The mechanism by which antibodies reduce $^1O_2$ is still being determined. However, the participation of a metal-mediated redox process has been largely discounted because the activity of the antibodies remains unchanged after exhaustive dialysis in PBS containing EDTA (4 mM). This leaves the intrinsic

15   ability of the amino acid composition of the antibodies themselves. Aromatic amino acids such as tryptophan (Trp) can be oxidized by $^1O_2$ via electron transfer (Grossweiner, L. I., Curr. Top. Radiat. Res. Q., 11, 141-199 (1976)). In addition, disulfides are sufficiently electron rich that they can also be oxidized (Bent. D. V. & Hayon, E., J. Am. Chem. Soc., 87, 2612-2619 (1975)). Therefore, there is the

20   potential that Trp residues and/or the intrachain or interchain disulfide bonds homologous to all antibodies are responsible for $^1O_2$ reduction. To both investigate to what extent this ability of antibodies is shared by other proteins and to probe the mechanism of reduction, a panel of other proteins was studied (FIG. 6).

Whereas other proteins can convert $^1O_2$ into $O_2^{\bullet-}$, in contrast to antibodies it

25   is by no means a universal property. RNase A and superoxide dismutase, which do not possess Trp residues but have several disulfide bonds, do not reduce $^1O_2$. Similarly, the Bowman-Birk inhibitor protein (Voss, R.-H., Ermler, U., Essen, L.-O., Wenzl, G., Kim, Y.-M. & Flecker, P., Eur. J. Biochem., 242, 122-131 (1996); Baek, J. & Kim, S., Plant Physiol., 102, 687 (1993)) that has seven disulfide bonds

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and zero Trp residues does not reduce $^1O_2$. In contrast, chick ovalbumin, which has only 2 Trp residues (Feldhoff, R. & Peters, T. J., Biochem. J., 159, 529-533 (1976)), is one of the most efficient proteins at reducing $^1O_2$.

Given the loss of antibody activity upon denaturation, the location of key

5    residues in the protein is likely to be more critical than their absolute number. Because the majority of aromatic residues in proteins are generally buried to facilitate structural stability (Burley, S. K. & Petsko, G. A., Science, 229, 23-28 (1985)), the nature of the reduction process was explored in terms of relative contribution of surface and buried residues by fluorescence-quenching experiments.

10    Aromatic amino acids in proteins are modified by the absorption of ultraviolet light, especially in the presence of sensitizing agents such as molecular oxygen or ozone (Foote, C. S., Science, 162, 963-970 (1968); Foote, C. S., Free Radicals Biol., 2, 85-133 (1976); Gollnick, K., Adv. Photochem., 6, 1-122 (1968)). Trp reacts with $^1O_2$ via a [2 + 2] cycloaddition to generate N-formylkynurenine or

15    kynurenine, which are both known to significantly quench the emission of buried Trp residues (Mach, H., Burke, C. J., Sanyal, G., Tsai, P.-K, Volkin, D. B. & Middaugh, C. R. in Formulation and Delivery of Proteins and Peptides, eds. Cleland, J. L. & Langer, R. (American Chemical Society, Denver, CO) (1994)). The intrinsic fluorescence of horse IgG is rapidly quenched to 30% of its original

20    value during a 40-min irradiation, whereas hydrogen peroxide generation is linear throughout ($r^2$ = 0.998) (FIG. 7). If the reduction of singlet oxygen is due to antibody Trp residues, then the solvent-exposed Trp seem to contribute to a lesser degree than the buried ones. This factor may help to explain why this ability is so highly conserved among antibodies. In greater than 99% of known antibodies there

25    are two conserved Trp residues, and they are both deeply buried: Trp-36 and Trp-47 (Kabat, E. A., Wu, T. T., Perry, H. M., Gottesman, K. S. & Foeller, C., Sequences of Proteins of Immunological Interest (U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Bethesda, MD) (1991)).

Throughout nature, organisms have defended themselves by production of relatively simple chemicals. At the level of single molecules, this mechanism has thought to be largely abandoned with the appearance in vertebrates of the immune system. It was considered that once a targeting device had evolved, the killing

5      mechanism moved elsewhere. The present results realign recognition with killing within the same molecule. In a certain sense this chemical immune system parallels the purely chemical defense mechanism of lower organisms, with the exception that a more sophisticated and diverse targeting element is added.

Given the constraints that an ideal killing system must use host molecules in

10     a localized fashion while minimizing self damage, one can hardly imagine a more judicious choice than $^1O_2$. Because one already has such a reactive molecule, it is important to ask what might be the advantage of its further conversion by the antibody. The key issue is that by conversion of the transient singlet oxygen molecule (lifetime 4 $\mu$s) into the more stable $O_2^{\bullet-}$, one now has access to hydrogen

15     peroxide and all of the toxic products it can generate. In addition, superoxide is the only molecular oxygen equivalent remaining at the end of the oxygen-scavenging cascade. Therefore, this "recycling" may serve as a crucial mechanism for potentiation of the microbicidal process. Another benefit of singlet molecular oxygen is that it is only present when the host is under assault, thereby making it an

20     "event-triggered" substrate. Also, because there are alternative ways to defend that use accessory systems, this chemical arm of the immune system might be silent under many circumstances. This said, however, there may be many disease states where antibody and singlet oxygen find themselves juxtaposed, thereby leading to cellular and tissue damage. Given that diverse events in man lead to the production

25     of singlet oxygen, its activation by antibodies may lead to a variety of diseases ranging from autoimmunity to reperfusion injury and atherosclerosis (Skepper et al., Microsc. Res. Tech., 42, 369-385 (1998)).

**Example II: Antibodies Catalyze the Oxidation of Water**

**Methods and Materials**

Crystallography: IgG 4C6 was digested with papain and the Fab' fragment purified using standard protocols (Harlow and Lane). The Fab' was crystallized

5      from 13-18% PEG 8 K, 0.2 M ZnAc, 0.1 M cacodylate, pH 6.5. Crystals were pressurized under xenon gas at 200 psi for two minutes (Soltis et al., J. Appl. Cryst., 30, 190, (1997)) and then flash cooled in liquid nitrogen. Data were collected to 2.0 Å resolution at SSRL BL9-2. The structure was solved by molecular replacement using coordinates from the native 4C6 structure, and xenon

10     atom sites were identified from strong peaks in the difference Fourier map. Refinement of the structure was done in CNS (Brünger et al., Acta. Crystallogr., D54, 905 (1998)) to final R = 23.1% and $R_{free}$ = 25.7%. The occupancies of the two xenon atoms were refined after fixing their B values fifty percent higher than the B factors of the immediately surrounding protein. The FIG.s were generated in

15     Bobscript (R.M. Esnouf, Acta Crystallog., D55, 938 (1999)).

Scanning of the Kabat database: The Kabat database of human and mouse sequences was analyzed to determine the number of Trp, Tyr, Cys, Met in their structures. Sequences were rejected if there were too many residue deletions or missing fragments. This allowed a high certainty analysis for 2068 of the 3894

20     sequences available. The values are reported as the mean totals with the range in parentheses of the $C_H$, $V_H$, $C_L$ and $V_L$ (κ and λ.) regions: Trp 15.5 (14 to 31), Tyr 30.4 (13 to 47), Cys 19.3 (15 to 29), Met 11.6 (7 to 32), His 13.3 (8 to 28). Grand total = 90.1 (49 to 167).

Inductively coupled plasma atomic emission spectroscopy: Inductively

25     coupled plasma atomic emission spectroscopy (ICP-AES) of mAb PCP21H3 was performed on a Varian, Axial Vista Simultaneous ICP-AES spectrometer. Mouse monoclonal antibody (PCP21H3) was exhaustively dialyzed into sodium phosphate buffered saline (PBS, 50 mM pH 7.4) with 20 mM EDTA. In a typical assay 300 µL of a 10.5 % $HNO_3$ solution was added to 100 µL of a 10 mg/mL antibody

solution and was incubated at 70°C for 14 hours. This solution was then diluted to 2 mL with MQH$_2$O and then analyzed by comparison to standards. ICP-AES analysis results are reported in parts per million (μg/mL): Ag 0.0026 (0.0072 atoms per antibody molecule); Al 0.0098 (0.113 atoms per antibody molecule); As 0.0062

5    (0.025 atoms per antibody molecule); Ba below level of detection; Ca 0.0355 (0.266 atoms per antibody molecule).

The high Ca concentration observed was a result of contamination of the phosphate buffer system utilized in the assay system. To investigate the effect of Ca(II) on the rate of antibody-mediated H$_2$O$_2$, the irradiation of antibody samples

10    was performed using the assay procedure outlined in the legend of FIG. 8A with the addition of varying concentrations of CaCl$_2$ (0 - 100 μM). The process was found to be independent of Ca(II) concentration; Cd 0.0007 (0.0187 atoms per antibody molecule); Ce 0.0012 (0.003 atoms per antibody molecule); Co 0.0013 (0.007 atoms per antibody molecule); Cr 0.0010 (0.006 atoms per antibody

15    molecule); Cu 0.0014 (0.007 atoms per antibody molecule); Fe 0.0089 (0.048 atoms per antibody molecule); Gd 0.0008 (0.001 atoms per antibody molecule); K 0.0394 (0.302 atoms per antibody molecule); La 0.0007 (0.002 atoms per antibody molecule); Li 0.0013 (0.056 atoms per antibody molecule); Mg 0.0027 (0.033 atoms per antibody molecule); Mn 0.0007 (0.004 atoms per antibody molecule);

20    Mo 0.0023 (0.007 atoms per antibody molecule); Na 102.0428 (1332 atoms per antibody molecule); Ni 0.0007 (0.004 atoms per antibody molecule); P 14.3521 (138.9 atoms per antibody molecule); Pb below level of detection; Rb 0.0007 (0.002 atoms per antibody molecule); Se below level of detection; V 0.0109 (0.019 atoms per antibody molecule); W 0.0119 (0.019 atoms per antibody molecule); Zn

25    0.0087 (0.040 atoms per antibody molecule).

Oxygen isotope experiments: In a typical experiment, a solution of antibody (6.7 μM, 100 μL) or non-immunoglobulin protein (50 μM, 100 μL) in PB (160 mM phosphate; pH 7.4) was lyophilized to dryness and then dissolved in H$_2$O$_2$ (100 μL, 98 %). Sodium chloride was excluded to minimize signal

suppression in the MS. The higher concentration of non-immunoglobulin protein was necessary to generate a detectable amount of $H_2O_2$ for the MS assay. This protein solution was irradiated on a UV-transilluminator under saturating $^{16}O_2$ aerobic conditions in a sealed quartz cuvette for 8 hours at 20EC. The $H_2O_2$

5    concentration was determined after 8 hours using the Amplex Red assay (Zhou et al., Anal. Biochem., 253, 162 (1997)). The sample was then filtered by centrifugation through a microcon (size-exclusion filter) to remove the protein and the $H_2O_2$ concentration re-measured. TCEP (freshly prepared 20 mM stock in $H_2{}^{18}O$) was added (ca. 2 mol eq relative to $H_2O_2$) and the solution was left to stand

10    at 37 °C for 15 minutes, after which time all the $H_2O_2$ had reacted. The TCEP solution in $H_2{}^{18}O$ was prepared fresh prior to every assay because $^{18}O$ is slowly incorporated into the carboxylic acids of TCEP (over days). During the time course of the assay, no incorporation of $^{18}O$ occurs due to this pathway. Furthermore, there is no incorporation of $^{18}O$ from $H_2{}^{18}O$ into the $^{16}O$ phosphine

15    oxide. The peak at 249 m/z is the (M-H)⁻ of TCEP. The peak at 249 is observed in all the MS because an excess of TCEP (twofold) relative to $H_2O_2$ is used in the assay.

The reproducibility of the $^{16}O/^{18}O$ ratio from protein samples lyophilized together was reasonable (±10 %). However, problems with removing protein-bound

20    water molecules during the lyophilization process meant that the observed ratios could vary between samples from different lyophilization batches by as much as 2:1 to 4:1 (when lyophilizing from $H_2{}^{16}O$). It is, therefore, important that rigorous lyophilization and degassing procedures were followed. In this regard, the $^{18}O_2$ and $H_2{}^{16}O$ experiments exhibited far less inter-assay variability due to the relative ease

25    of removing protein-bound oxygen molecules.

Antibodies from different species give similar ratios within the experimental constraints detailed below: $^{16}O{:}^{18}O$: WD1-6G6 mIgG (murine) 2.1:1; poly-IgG (horse) 2.2:1; poly-IgG(sheep) 2.2:1; EP2-19G2 mIgG (murine) 2.1: 1; CH2-5H7 mIgG (murine) 2.0:1; poly-IgG (human) 2.1:1. Ratios were based on the

mean value of duplicate determinations except for poly-IgG (horse), which was the mean value of ten measurements. All assays and conditions were as described above.

In a typical experiment, a solution of sheep or horse poly-IgG (6.7 μM, 100 μL) in PB (160 mM phosphate; pH 7.4) was degassed under an argon atmosphere for 30 min. This solution was then saturated with $^{18}O_2$ (90 %) and irradiated as described above. Assays and procedures are then as described herein.

Assay for $H_2O_2$ production as a function of the efficiency of $^1O_2$ formation via $^3O_2$ sensitization with hematoporphyrin IX: The assay is a modification of a procedure developed by H. Sakai and co-workers, Proc. SPIE-Int. Soc. Opt. Eng., 2371, 264 (1995). In brief, the horse poly-IgG (1 mg/mL) in PBS (50 mM, pH 7.4) and hematoporphyrin IX (40 μM) was irradiated with white light from a transilluminator. Aliquots were removed (50 μL) and the concentration of $H_2O_2$ and 3-aminophthalic acid measured simultaneously. $H_2O_2$ concentration was measured by the amplex red assay (Zhou et al., Anal. Biochem., 253, 162 (1997)). 3-Aminophthalic acid concentration was measured by reversed-phase HPLC on a Hitachi D4000 series machine with an Adsorbosphere-C18 column, a UV detector at 254 nm, and a mobile phase of acetonitrile/water (0.1% TFA) of 18:82 at 1 mL/min (retention time of luminol = 7.4 min and of 3-aminophthalic acid = 3.5 min). The concentrations of luminol and 3-aminophthalic acid were determined by comparison of peak height and area to control samples. The experimental data yielded the amount of $^1O_2$ formed by hematoporphyrin IX (being directly proportional to the amount of 3-aminophthalic acid formed) and the amount of $H_2O_2$ formed by the antibody. There was no significant amount of $^1O_2$ formed by antibodies without hematoporphyrin IX in white light.

Any concerns that the amplex red assay was detecting protein-hydroperoxide derivatives in addition to $H_2O_2$ were discounted because the apparent $H_2O_2$ concentration measured using this method was independent of

whether irradiated protein was removed from the sample (by size-exclusion filtration).

Quantum Chemical Methods: All QC calculations were carried out with Jaguar [Jaguar 4.0, Schrödinger, Inc. Portland, Oregon, 1998. See B. H. Greeley, T.

5    V. Russo, D. T. Mainz, R. A. Friesner, J.-M. Langlois, W. A. Goddard III, R. E. Donnelly, J. Chem. Phys., 101, 4028 (1994)] using the B3LYP flavor of density functional theory (DFT) [J. C. Slater in Quantum Theory of Molecules and Solids, Vol. 4: The Self-Consistent Field of Molecules and Solids, McGraw Hill, New York, (1974)], that included the generalized gradient approximation and exact

10   exchange. The 6-31G** basis set was used on all atoms. All geometries were fully optimized. Vibrational frequencies were calculated to ensure that each minimum was a true local minimum (only positive frequencies) and that each transition state (TS) had only a single imaginary frequency (negative eigenvalue of the Hessian). Such QC calculations have been demonstrated to have an accuracy of about 3

15   kcal/mol for simple organic molecules. Non-closed shell molecules such as $O_2$ and $^3O_2$ were expected to have larger errors. However, such errors were expected to be systematic such that the mechanistic implications of the QC results would be correct. All energetics were reported in kcal/mol without correcting for zero point energy or temperature.

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## Results and Discussion

Antibodies are capable of generating hydrogen peroxide ($H_2O_2$) from singlet molecular oxygen ($^1O_2$). However, it was not known until reported herein, that the process was catalytic. It is now shown that antibodies are unique as a class

25   of proteins in that they can produce up to 500 mole equivalents of $H_2O_2$ from $^1O_2$, without a reduction in rate, in the absence of any discernible cofactor and electron donor. Based on isotope incorporation experiments and kinetic data, it is proposed that antibodies are capable of facilitating an unprecedented addition of $H_2O$ to $^1O_2$ to form $H_2O_3$ as the first intermediate in a reaction cascade that eventually leads to

$H_2O_2$. X-ray crystallographic studies with xenon point to conserved oxygen binding sites within the antibody fold where this chemistry could be initiated. These findings suggest a unique protective function of immunoglobulins against $^1O_2$ and raise the question of whether the need to detoxify $^1O_2$ has played a decisive role in

5     the evolution of the immunoglobulin fold.

Antibodies, regardless of source or antigenic specificity, generate hydrogen peroxide ($H_2O_2$) from singlet molecular oxygen ($^1O_2$) thereby potentially aligning defensive recognition and killing within the same molecule (Wentworth et al., Proc. Natl. Acad. Sci. U.S.A., 97, 10930 (2000)). Given the potential chemical and

10     biological significance of this discovery, the mechanistic basis and structural location within the antibody of this process has been investigated. These combined studies reveal that, in contrast to other proteins, antibodies may catalyze an unprecedented set of chemical reactions between water and singlet oxygen.

Kinetic studies. Long term UV irradiation studies reveal that

15     antibody-mediated $H_2O_2$ production is a much more efficient process than is the case for the non-immunoglobulin proteins (FIG. 8A). Typically antibodies exhibit linearity in $H_2O_2$ formation for up to 40 mole equivalents of $H_2O_2$ before the rate begins to decline asymptotically (FIG. 8B). By contrast, non-immunoglobulin proteins display a short 'burst' of $H_2O_2$ production followed by quenching as

20     photo-oxidation occurs (FIG. 8A).

In contrast to other proteins, antibodies are able to resume photo-production of $H_2O_2$ at the same initial rate as at the start of the experiment if the $H_2O_2$ generated during the assay is removed by catalase, as shown for murine monoclonal IgG PCP21H3 (FIG. 8C). This profile of continued linear production

25     of $H_2O_2$ after catalase-mediated destruction of $H_2O_2$ was conserved for all antibodies assayed. Thus, the $H_2O_2$ that accumulated during the process was inhibiting (reversibly) its own formation. The apparent $IC_{50}$ was estimated as 225 $\mu M$ (FIG. 8D).

Inhibition of the catalytic function of an enzyme either by substrates, transition state analogs or reaction products is often taken as strong evidence for an active site phenomenon. It has already been noted that the antibody-mediated photo-production of $H_2O_2$ is saturable with molecular oxygen ($K_mapp$ ($O_2$) 187

5    μM) (Wentworth et al., Proc. Natl. Acad. Sci. U.S.A., 97, 10930 (2000)). This formal product inhibition of $H_2O_2$ provides further evidence for such a binding site phenomenon.

An earlier report concerning the photo-production of $H_2O_2$ by antibodies did not probe the maximum amount of $H_2O_2$ that could be generated (Wentworth et

10    al., Proc. Natl. Acad. Sci. U.S.A., 97, 10930 (2000)). This issue has been examined by repetitive cycles of UV irradiation of antibody samples followed by removal of the generated $H_2O_2$ by catalase (FIG. 8C shows two such cycles). In one series of experiments, the cycle of UV-irradiation and addition of catalase was carried out for up to 10 cycles (horse poly IgG in PBS, pH 7.4). During these experiments >

15    500 mole equivalents (equiv.) of $H_2O_2$ were generated, with only a slight reduction in the initial rate being observed. Beside antibodies, the only other protein that was found thus far to generate $H_2O_2$ in such an efficient and long-term manner was the αβ T cell receptor (αβ TCR) (FIG. 8F). Interestingly, the αβ TCR shares a similar arrangement of its immunoglobulin fold domains with antibodies (Garcia et al.,

20    Science, 274, 209 (1996)). However, possession of this structural motif seems not necessarily to confer a $H_2O_2$-generating ability on proteins as demonstrated by β2-microglobulin, which does not generate $H_2O_2$ even though it is a member of the immunoglobulin superfamily (Welinder et al., Mol. Immunol., 28, 177 (1991)).

The antibody structure is remarkably inert against the oxidizing effects of

25    $H_2O_2$. When exposed to standard UV irradiation conditions for 6 hours in the presence of $H_2O_2$ (at a concentration high enough to fully inhibit $H_2O_2$ production), a polyclonal horse IgG antibody sample becomes fully active once the inhibitory $H_2O_2$ has been destroyed by catalase (FIG. 8E). The ability to continue $H_2O_2$ production for long periods at a constant rate, even after exposure to $H_2O_2$, reveals

a remarkable, and hitherto unnoticed, resistance of the antibody structural fold to both chemical and photo-oxidative modifications suffered by other proteins. SDS-PAGE gel analysis of antibody samples after UV irradiation under standard conditions for 8 hours reveals neither significant fragmentation nor agglomeration

5    of the antibody molecule.

To ensure that there was no change in the protein structure in the presence of $H_2O_2$ (that may be contributing to the apparent inhibitory effect of $H_2O_2$) even at the level of side-chain position, x-ray crystal structures of Fab 4C6 were determined in the presence and absence of $H_2O_2$. Fab 4C6 was selected because its

10    native crystals diffract to a higher resolution than any other published antibody (~1.3 D). The root mean square difference (RMSD) of key structural parameters was compared for the 4C6 structure before and after a soak experiment with 3 mM $H_2O_2$. RMSD of all atoms = 0.412 D, RMSD C$\alpha$ atoms = 0.327 D, RMSD main chain atoms = 0.328 D, RMSD side-chain atoms = 0.488 D. The overlayed native

15    and $H_2O_2$-treated structures of murine Fab 4C6 (Li et al., J. Am. Chem. Soc., 117, 3308 (1995)) were superimposable, reinforcing the evidence of stability of the antibody fold to $H_2O_2$ (FIG. 9).

An action spectrum of the antibody-mediated photo-production of $H_2O_2$ and the corresponding absorbance spectrum of the antibody protein for the same

20    wavelength range (260 - 320 nm) are juxtaposed in FIG. 10. The two spectra are virtually superimposable with maximal efficiency of $H_2O_2$ production being observed at an excitation wavelength that coincides with the UV absorbance maxima of tryptophan in proteins.

Probing the efficiency of $H_2O_2$ production by horse IgG as a function of the

25    efficiency of $^1O_2$ formation via $^3O_2$ sensitization with hematoporphyrin IX ($\varphi_A$ = 0.22 in phosphate buffer pH 7.0) and visible light reveals that for every 275 ± 25 mole equivalents of $^1O_2$ generated by sensitization, 1 mole equivalent of $H_2O_2$ is generated by the antibody molecule (Wilkinson et al., J. Phys. Chem. Ref. Data, 22, 113 (1993); Sakai et al., Proc. SPIE-Int. Soc. Opt. Eng., 2371, 264 (1995)).

The question of the electron source. The mechanism problem posed by the antibody-mediated $H_2O_2$ production from singlet oxygen can be divided into two sub-problems: one referring to the electron source for the process and the other concerning the chemical mechanism of the process. Given that the conversion of

5 $^1O_2$ to $H_2O_2$ requires two mole equivalents electrons, the fact that antibodies can generate > 500 equivalents of $H_2O_2$ per equivalent of antibody molecule raises an acute electron inventory problem. The search for this electron source began with the most distinct possibilities. Electron transfer through proteins can occur with remarkable facility and over notably large distances (Winkler et al., Pure & Appl.

10 Chem., 71, 1753 (1999); Winkler, Curr. Opin. Chem. Biol., 4, 192 (2000)). The first electron source considered was a collection of the residues typically implicated as electron donors and cited in normal protein photo-oxidation processes. The nearly constant rate of $H_2O_2$ production by antibodies and αβ-TCR during repetitive cycles of irradiation and catalase treatment (FIG. 8C and 8E) argued

15 against such a mechanism because a marked reduction of rate would have to accompany $H_2O_2$ production as the residues capable of being oxidized became exhausted. This reduction of rate would be further exacerbated because the redox potentials of the remaining unoxidized residues would have to rise as the protein becomes more positively charged.

20 Normal protein photo-oxidation is a complex cascade of processes that leads to the generation of $^1O_2$ and other reactive oxygen species (ROS), such as superoxide anion ($O_2^{\bullet-}$), peroxyl radical ($HO_2^{\bullet}$) and $H_2O_2$ (Foote, Science, 162, 963 (1968)). Present mechanistic thinking links the sensitivity of proteins to photo-oxidation with up to five amino acids: tryptophan (Trp), tyrosine (Tyr),

25 cysteine (and cystine), methionine (Met), and histidine (His) (Straight and Spikes, in Singlet $O_2$, A.A. Frimer, Ed. (CRC Press, Inc., Boca Raton, Florida, 1985), vol IV9, pp. 91-143; Michaeli and Feitelson, Photochem. Photobiol., 59, 284 (1994)). The photo-production of $H_2O_2$ by Trp and molecular oxygen is a well-characterized process that involves, at least in part, the formation and reduction of $^1O_2$ to $O_2^{\bullet}$ that

spontaneously dismutates into $H_2O_2$ and $^3O_2$ (McMormick and Thompson, J. Am.
Chem. Soc., 100, 312 (1978)). Tryptophan, both as an individual amino-acid and
as a constituent of proteins, is particularly sensitive to near-UV irradiation
(300-375 nm) under aerobic conditions, owing to its conversion to N,N-

5      formylkynurenine (NFK) that is a particularly effective near-UV ($\lambda_{max}$ 320 nm)
photosensitizer (Walrant and Santus, Photochem. Photobiol., 19, 411 (1974)).
However, Trp photo-oxidation is accompanied by sub-stoichiometric production of
$H_2O_2$ (ca. 0.5 mole equivalents) during near-UV irradiation (FIG. 11A)
(McMormick and Thompson, J. Am. Chem. Soc., 100, 312 (1978)) and the most

10     efficient non-immunoglobulin protein at $H_2O_2$ photo-production, β-galactosidase,
generates only 5.9 mol eq. of $H_2O_2$ from its 39 Trp residues (FIG. 8A) (Fowler and
Zabin, J. Biol. Chem., 253, 5521 (1978)).

         Scanning of the Kabat database of human and mouse antibody heavy- and
light-chain sequences (2068 of 3894 sequences were analyzed) revealed that

15     antibodies rarely have more than 15 Trp residues in their entire structure (mean
value = 15.5 with a range of 14 to 31 Trp residues)(Kabat et al., Sequences of
Proteins of Immunological Interest (US Department of Health and Human Services,
Public Health Service, NIH, ed. 5th, 1991); Martin, PROTEINS: Struct., Funct. and
Genet., 25, 130 (1996)). In fact, even if all of the amino acids that are implicated in

20     protein photo-oxidation processes vide supra are collectively involved in
antibody-mediated $H_2O_2$-production, there is still an insufficient number of these
residues (mean value = 90.1 with a range of 49 to 167 reactive residues) to account
for the 500 mole equivalents of $H_2O_2$ generated.

         The potential of chloride ion (present at 150 mM in PBS) as a reducing

25     equivalent was then investigated given that chloride ion is known to be a suitable
electron source for photo-production of $H_2O_2$ via a triplet excited state of an
anthraquinone (Scharf and Weitz, Symp. Quantum Chem. Biochem., Jerusalem vol.
12 (Catal. Chem. Biochem.: Theory Exp.), pp. 355-365 (1979)). This possibility
was quickly discounted when the rate of $H_2O_2$ production by immunoglobulins was

found to be independent of chloride ion concentration (FIG. 11B).

The possible role of metal ions was investigated. While such ions could hardly be present in antibodies in such amounts that they could serve as an electron source, trace amounts of them might play a central role as catalytic redox centers.

5    Experiments were performed that, for all practical purposes, allowed the involvement of trace metals in this process to be ruled out. For example, the rate of antibody-mediated photo-production of $H_2O_2$ was unchanged before and after exhaustive dialysis of antibody samples with EDTA-containing buffer (FIG. 11C). After EDTA treatment of antibody samples, ICP-atomic emission spectroscopy

10   (AES) revealed the presence of trace metal ions remaining in amounts that are far below parts per million. For a trace metal to be implicated in this reaction it must be common to all antibodies because all antibodies assayed have this intrinsic ability. It is generally accepted that metal-binding is not an implicit feature of antibodies and is consistent with an analysis of antibody crystals performed by the

15   inventors as well as the approximate 300 antibody structures available on the Brookhaven database.

All of the observations thus far forcibly pointed towards the need to identify an electron source that would not involve deactivation of the protein catalyst and that could account for the high turnover numbers and hence, for a *quasi* unlimited

20   source of electrons.

A more broad consideration of the chemical potential of $^1O_2$ was done. The participation of this energized form of molecular oxygen in the antibody-mediated mechanism was clearly inferred from a previous report (Wentworth et al., Proc. Natl. Acad. Sci. U.S.A., 97, 10930 (2000)). In brief, the antibody-mediated rate of

25   $H_2O_2$ photo-production is increased in $D_2O$ and reduced in the presence of the $^1O_2$ quencher, sodium azide. Furthermore, antibodies have been shown to generate $H_2O_2$ via sensitization of $^3O_2$ with hematoporphyrin IX in visible light, and in the dark with the endoperoxide of disodium 3N,3N-(1,4-naphthylidene) dipropionate (a chemical $^1O_2$ source). The involvement of $^1O_2$ is also consistent with the close

similarity of the action spectrum of antibody-mediated $H_2O_2$ production and the absorbance spectrum of antibody constituent tryptophans (FIG. 10).

Given that the known chemistry of $^1O_2$ can be conceptualized as the chemistry of the super-electrophile "dioxa-ethene" (Foote, Acc. Chem. Res., 1, 104

5    (1968), the heretofore unknown possibility was considered that a molecule of water may, in the presence of an antibody, add as a nucleophile to $^1O_2$ and form $H_2O_3$ as an intermediate. Thus, water becoming oxidized to $H_2O_2$ would fulfill the role of the electron source.

Oxygen isotope experiments were undertaken to test the hypothesis of an

10   antibody-catalyzed photo-oxidation of $H_2O$ by $^1O_2$ through determination of the source of oxygen found in the $H_2O_2$. Contents of $^{16}O/^{18}O$ in $H_2O_2$ were measured by modification of a standard $H_2O_2$ detection method (Han et al., Anal. Biochem., 234, 107 (1996)). Briefly, this method involves reduction with tris carboxyethyl phosphine (TCEP), followed by mass-spectral (MS) analysis of the corresponding

15   phosphine oxides (FIG. 12).

These experiments revealed that UV-irradiation of antibodies, in the presence of oxygen, leads to oxygen incorporation from water into $H_2O_2$ (FIGs. 12A and 12B). The relative abundance of the $^{16}O/^{18}O$ ratio observed in the MS of the phosphine oxide after irradiation of sheep poly-IgG under conditions of

20   saturating $^{16}O_2$ concentration in a solution of $H_2^{18}O$ (98 % $^{18}O$) phosphate buffer (PB) is $2.2 \pm 0.2{:}1$ (FIG. 12A). When the converse experiment is performed, with an $^{18}O$ enriched molecular oxygen mixture (90 % $^{18}O$) in $H_2^{16}O$ PB, the reverse ratio $(1{:}2.0 \pm 0.2)$ is observed (FIG. 12B). These values of the ratios exhibit good reproducibility (+ 10 %, $n = 10$) and are found for all antibodies studied.

25   The following control experiments were performed. First, under conditions of $^{16}O_2$ and $H_2^{16}O$, irradiation of poly-IgG (horse) generated $H_2^{16}O_2$ (FIG. 12C). There is no incorporation of $^{18}O$ when $H_2^{16}O_2$ (400 μM in PB, pH 7.0) itself is irradiated for 4 hours in $H_2^{18}O$. This result alleviates concerns that $^{18}O$ incorporation into $H_2O_2$ may be occurring via either an acid-catalyzed exchange
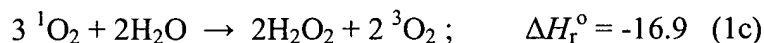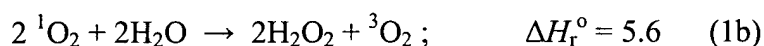
with water or by a mechanism that involves homolytic cleavage of $H_2{}^{16}O_2$ and recombination with $H^{18}O^\bullet$ from water. To check the possibility that antibodies may catalyze both the production of $H_2{}^{16}O_2$ and its acid-catalyzed exchange with $H_2{}^{18}O$, the isotopic exchange of $H_2{}^{16}O_2$ (200 μM) in $H_2{}^{16}O_2$ (98 % $^{18}O$) was

5    observed in the presence of sheep poly-IgG (6.7 μM) after UV-irradiation under an inert atmosphere. Only a trace of incorporation of $^{18}O$ into $H_2{}^{16}O_2$ was observed (FIG. 12D).

Isotope experiments were also performed with β-galactosidase, the most efficient non-immunoglobulin protein at generating $H_2O_2$, as well as

10    3-methylindole. In both cases, photo-oxidation led to negligible $^{18}O$ incorporation into the $H_2O_2$ (FIGs. 12E and 12F), illustrating that the indole ring itself and tryptophan residues in this protein are behaving simply as reductants of $^1O_2$.

This view is further supported by observations that irradiation of 3-methylindole generates $H_2O_2$ but the $H_2O_2$ generated does not include oxygen

15    incorporation from $H_2{}^{18}O$. The same experiment performed with tryptophan does give rise to exchange with a ratio $^{16}O/^{18}O$ 1.2:1. This result is thought to be due to the ammonium functionality acting as an intramolecular general acid that protonates the internal oxygen of a diastereomeric mixture of 3'-hydroperoxides. It should be noted that while this is interesting from a chemical point of view, it

20    cannot account for the catalytic production of $H_2O_2$ by antibodies both because it is a stoichiometric process and Trp residues in proteins do not possess a free ammonium group.

The chemical mechanism. All antibodies studied can catalyze the oxidation

25    of water by singlet oxygen. The thermodynamic balance between reactants and products for the oxidation of $H_2O$ by $^1O_2$ (heat of reaction, $\Delta H_r = +28.1$ kcal/mol) (D.R. Lide, in Hanbook of Chemistry and Physics, 73$^{rd}$ ed. (CRC, 1992)), demands a stoichiometry in which more than one molecule of $^1O_2$ would have to participate per molecule of oxidized water during its conversion into two molecules of $H_2O_2$.
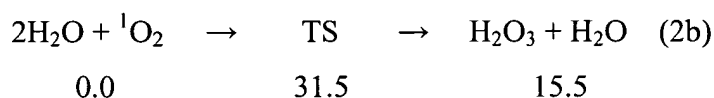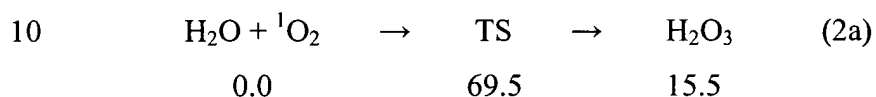
This stoichiometry assumes that no further light energy before that involved in the production of singlet from triplet oxygen is participating in the process. Qualitative chemical reasoning on hypothetical mechanistic pathways, together with thermodynamic considerations, makes the likely overall stoichiometries as in either

5 equations 1b or c (all energetics are calculated from gas phase experimental heats of formation and are reported in kcal/mol):
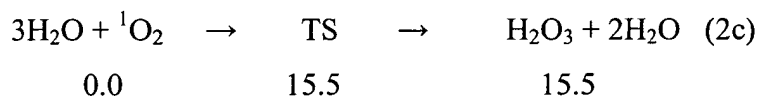
$$^1O_2 + 2H_2O \rightarrow 2H_2O_2 ; \qquad \Delta H_r^o = 28.1 \quad (1a)$$

$$2\,^1O_2 + 2H_2O \rightarrow 2H_2O_2 + {}^3O_2 ; \qquad \Delta H_r^o = 5.6 \quad (1b)$$

$$3\,^1O_2 + 2H_2O \rightarrow 2H_2O_2 + 2\,^3O_2 ; \qquad \Delta H_r^o = -16.9 \quad (1c)$$

10 A recent report of a transition metal-catalyzed conversion of $^1O_2$ and water into hydrogen peroxide, via a tellurium-mediated redox process (Detty and Gibson, J. Am. Chem. Soc., 112, 4086 (1990)), provides experimental evidence for a process in which $^1O_2$ and $H_2O$ can be converted into $H_2O_2$ and, hence that the energetic demands of this process can be overcome. It is thought that the mechanism for the

15 antibody-mediated photo-oxidation process involves the addition of a molecule water to a molecule of $^1O_2$ to form dihydrogen trioxide as the first intermediate on the way to $H_2O_2$. The antibody's function as a catalyst would have to be the supply of a specific molecular environment that would stabilize the critical intermediate relative to its reversible formation and, or, would accelerate the consumption of the

20 intermediate by channeling its conversion to $H_2O_2$. An essential feature of such an environment might consist of a special constellation of organized water molecules at an active site conditioned by an antibody-specific surrounding.

While $H_2O_3$ has not yet been detected in biological systems, its chemistry *in vivo* has been a source of considerable speculation and its *in vitro* properties have

25 been the subject of numerous experimental and theoretical treatments (C. Deby, La Recherche, 228, 378 (1991); Sawyer, in Oxygen Chemistry (Oxford University Press, Oxford, 1991); Cerkovnik and Plesnicar, J. Am. Chem. Soc., 115, 12169 (1993); Vincent and Hillier, J. Phys. Chem., 99, 3109 (1995); Plesnicar et al., Chem. Eur. J., 6, 809 (2000); Corey et al., J. Am. Chem. Soc., 108, 2472 (1986); Koller and

Plesnicar, J. Am. Chem. Soc., 118, 2470 (1996); Cacace et al., Science, 285, 81

(1999)). Plesnicar and co-workers have shown that $H_2O_3$, reductively generated

from ozone, decomposes into $H_2O$ and $^1O_2$ (Koller and Plesnicar, J. Am. Chem.

Soc., 118, 2470 (1996)). Applying the principle of microscopic reversibility, it was

5    surmised that the reverse reaction should also be catalyzed by one or more

molecules of water. To delineate plausible reaction routes and energetics of such a

process, first principles quantum chemical (QC) methods were used (B3LYP

Density Functional Theory) as described herein. The results are illustrated in

equations 2a-c (all energetics are in kcal/mol):

10
$$H_2O + {}^1O_2 \quad \rightarrow \quad TS \quad \rightarrow \quad H_2O_3 \qquad (2a)$$
$$0.0 \qquad\qquad 69.5 \qquad\quad 15.5$$

$$2H_2O + {}^1O_2 \quad \rightarrow \quad TS \quad \rightarrow \quad H_2O_3 + H_2O \quad (2b)$$
$$0.0 \qquad\qquad 31.5 \qquad\quad 15.5$$

15

$$3H_2O + {}^1O_2 \quad \rightarrow \quad TS \quad \rightarrow \quad H_2O_3 + 2H_2O \quad (2c)$$
$$0.0 \qquad\qquad 15.5 \qquad\quad 15.5$$

The direct reaction of water and $^1O_2$ to give $H_2O_3$ is quite unfavorable, with

20   an activation barrier of 70 kcal/mol (Eqn. 2a). However, with the addition of a

second or third water molecule a concerted process is found that decreases the

activation barrier to 31.5 and 15.5 kcal/mol respectively. Indeed these additional

waters do play the role of a catalyst (in equation. 2b the H of the 2nd water goes to

the product HOOOH, simultaneous with the H of the 1st water replacing it). These

25   barriers are small compared with the first HO bond energy of water (119 kcal/mol)

and the bond energy of $^1O_2$ (96 kcal/mol). Note that the reverse reaction in Eqn. 2b

and Eqn. 2c has a barrier of only 15.5 or 0 kcal/mol respectively, suggesting that

$H_2O_3$ is not stable in bulk water or water rich systems. Thus, the best site within the

antibody structure for producing and utilizing $H_2O_3$ is expected to be one in which

there are localized waters and water dimers next to hydrophobic regions without such waters.

The $^{16}O/^{18}O$ ratio in the phosphine oxide derived from the antibody-catalyzed photo-oxidation of water poses a significant constraint to the selection of
5   reaction paths by which this primary intermediate $H_2O_3$ would to convert to the final product $H_2O_2$. The ratio is primarily determined by the number of $^1O_2$ molecules that chemically participate in the production of two moles of $H_2O_2$ from two moles of $H_2O$ as well as by mechanistic details of this process. A ratio of 2.2:1 would coincide exactly with the value predicted for certain mechanisms in which two
10   molecules of $^1O_2$ and two molecules of $H_2O$ are transformed into two molecules of $H_2O_2$ and one molecule of molecular oxygen (which would have to be $^3O_2$ for thermodynamic reasons). An example of such a mechanism is an $S_N2$-type disproportionation of two molecules of $H_2O_3$ into $H_2O_4$ and $H_2O_2$, followed by the decomposition of the former into $H_2O_2$ and $^3O_2$. The complex problem of defining
15   theoretically feasible reaction pathways for the conversion of $H_2O_3$ into $H_2O_2$ with or without the participation of $^1O_2$ has been tackled in a systematic way using quantum chemical methods (B3LYP Density Functional Theory). These studies show extensive docking calculations of $H_2O_3$ and the transition states for its formation and conversion into $H_2O_2$ to a number of proteins. Indeed there are
20   unique sites of stabilizing these species in a region of antibodies (and the $\alpha\beta$-T cell receptor) in a region with isolated waters and next to hydrophobic regions. This extended study revealed the potential existence of a whole spectrum of theoretically feasible chemical pathways for the $H_2O_3$ to $H_2O_2$ conversion.

Structural studies of xenon binding to antibodies. Given the conserved
25   ability of antibodies, regardless of origin or antigen specificity, or of the $\alpha\beta$- TCR to mediate this reaction, X-ray structural studies were instigated to search for a possible conserved reaction site within these immunoglobulin fold proteins. A key constraint for any potential locus is that molecular oxygen (either $^1O_2$ or triplet with a potential sensitizing residue in proximity, preferably tryptophan) and water must

be able to co-localize, and the transition-states and intermediates along the pathway must be stabilized either within the site or in close proximity.

There is strong evidence to support the notion that Xe and $O_2$ co-localize in the same cavities within proteins (Tilson et al., J. Mol. Biol., 199, 195 (1988);

5    Schoenborn et al., Nature, 207, 28 (1965)). Accordingly, xenon gas was used as a heavy atom tracer to locate cavities within the murine monoclonal antibody 4C6 that may be accessible to molecular oxygen (Li et al., J. Am. Chem. Soc., 117, 3308 (1995)).

Three xenon sites were identified (FIG. 13A), and all occupy hydrophobic

10    cavities as observed in other Xe-binding sites in proteins (Scott and Gibson, Biochemistry, 36, 11909 (1997); Prangé et al., PROTEINS: Struct., Funct. and Genet., 30, 61 (1998)). Superposition of the refined native and Xe-derivatized structures shows that, aside from addition of xenon, there is little discernible change in the protein backbone or side chain conformation or in the location of bound water

15    molecules.

The xenon I binding site (Xe1 site) has been analyzed here in more detail because it is conserved in all antibodies and the αβ TCR (FIG. 13B). Xe1 is in the middle of a highly conserved region between the β-sheets of $V_L$, 7 Å from an invariant Trp. The Xe1 site is sandwiched between the two β-sheets that comprise

20    the immunoglobulin fold of the $V_L$, approximately 5 Å from the outside molecular surface. Xenon site two (Xe2) sits at the base of the antigen binding pocket directly above several highly conserved residues that form the structurally conserved interface between the heavy and light chains of an antibody (FIG. 13A). The residues in the $V_L$ $V_H$ interface are primarily hydrophobic and include conserved

25    aromatic side chains, such as $Trp^{H109}$.

The contacting side chains for Xe1 in Fab 4C6 are $Ala^{L19}$, $Ile^{L21}$, $Leu^{L73}$, and $Ile^{L75}$, which are highly conserved aliphatic side chains in all antibodies (Kabat et al., Sequences of Proteins of Immunological Interest (US Department of Health and Human Services, Public Health Service, NIH, ed. 5th, 1991)). Additionally, only

slight structural variation was observed in this region in all antibodies surveyed. Notably, several other highly conserved and invariant residues are in the immediate vicinity of this xenon site, including $Trp^{L35}$, $Phe^{L62}$, $Tyr^{L86}$, $Leu^{L104}$, and the disulfide-bridge between $Cys^{L23}$ and $Cys^{L88}$. $Trp^{L35}$ stacks against the disulfide-

5     bridge and is only 7 Å from the xenon atom. In this structural context, $Trp^{L35}$ may be a putative molecular oxygen sensitizer, since it is the closest Trp to Xe1. Comparison with the 2C αβ TCR structure and all available TCR sequences shows that this Xe1 hydrophobic pocket is also highly conserved in TCRs (FIG. 5B) (Garcia, Science, 274, 209 (1996)).

10     Human β₂-microglobulin, which does not generate $H_2O_2$, does not have the same detailed structural characteristics that define the antibody Xe1 binding pocket, despite its overall immunoglobulin fold. Also, β₂-microglobulin does not contain the conserved Trp residue that occurs there in both antibodies and TCRs. If $Trp^{L35}$ (antibodies) or $Trp^{\alpha34}$ (TCR) is the oxygen sensitizer, the lack of a corresponding

15     Trp in β₂-microglobulin may relate to the finding that it does not catalyze the oxidation of water.

Thus, the xenon experiments have identified at least one site that is both accessible to molecular oxygen and is in a conserved region ($V_L$) in close proximity to an invariant Trp; an equivalent conserved site is also possible in the fold of $V_H$.

20     The structure and sequence around the Xe1 site is almost exactly reproduced in the $V_H$ domain by the pseudo two-fold rotation axis that relates $V_L$ to $V_H$. Although a xenon binding-site was not located in this domain, it is thought that molecular oxygen can still access the corresponding cavity in $V_H$. The proposed heavy chain xenon site may not have been found because the crystals were pressurized for only

25     two minutes, which may have been insufficient time to establish full equilibrium, or simply because xenon is too large compared to oxygen for the corresponding cavity on the $V_H$ side, or due to crystal packing. In other antibody experiments, Xe binding sites were found in only one of the two molecules of the asymmetric unit that suggests that crystal packing can modulate access of Xe in crystals. Analysis of

the sequence and structure around these sites shows that they are highly conserved in both antibodies and TCRs thus providing a possible understanding of why the Ig-fold in antibodies and the TCR can be involved in this unusual chemistry.

5 Antibodies are unique among proteins in their ability to catalytically convert $^1O_2$ into $H_2O_2$. It is thought that this process participates in killing by event-related production of $H_2O_2$. Alternatively, antibodies can fulfill the function of defending an organism against $^1O_2$. This would require the further processing of hydrogen peroxide into water and triplet oxygen by catalase.

## EXAMPLE III: Antimicrobial Activity of Antibodies

10 This Example illustrates that antibodies directed against bacteria can kill those bacteria by generating reactive oxygen species.

## Materials and Methods

### Antibody and Cell Preparations

15 Sheep (31243) and horse (31127) polyclonal IgG were obtained from Pierce and used without further purification. The *E. coli* O112a,c-specific murine monoclonal antibody (15404) was obtained from QED biosciences and was used without further purification. The *E. coli* non-specific murine monoclonal antibodies 33F12 and 84G3 were obtained from the Scripps Hybridoma lab and used at > 98 %

20 purity (based on SDS-PAGE analysis). Monoclonal 33F12 is a murine monoclonal IgG that catalyzes the aldol reaction. Wagner et al., *Science* **270**, 1797 (1995). *E. coli* XL1-B was obtained from Stratagene. *E. coli* O112a,c (ATCC 12804) is an enteroinvasive strain which can infect malnourished and immuno-compromised individuals. L. Siegfried, M. Kmetove, H. Puzova, M. Molokacova, J. Filka, *J.*

25 *Med. Microbiol.* **41**, 127 (1994).

The following antibody preparations were prepared in-house by the following methods.

**Rabbit polyclonal IgG specific for *E. coli* XL-1 blue.**

On the day of immunization (Day 0), New Zealand White rabbits, (2.5 kg) were pre-bled 10 ml from each ear and then injected subcutaneously with heat killed (65 °C, 15min), chemically competent *E. coli* XL-1 ($OD_{600}$=1) (650 µl and 350 µl of

5   phosphate buffered saline, PBS ph 7.4). Fourteen days after immunization (Day 14), the rabbits received a second injection in the same manner as the first. Twenty eight days after immunization (Day 28), the rabbits received a third injection in the the same manner as the first and second injections. At thirty five days after immunization (Day 35), the rabbits were bled 50 ml from an ear. At forty two days

10  after immunization, (Day 42), the rabbits were bleed 50 ml from an ear.

Sera were allowed to stand at room temperature for 1-2 h, then placed at 4 °C overnight and spun at 2500-3500 rpm for 15 min. The supernatants were transferred to a new round bottom tube (50 ml) and spun at 9-10 K rpm for 15min. These supernatants were transferred to a clean conical (50 ml) tube and stored at –

15  10 °C. Sera were then tested by ELISA (see below), diluted 1:1 in PBS and then filtered through a 0.2 µM filter. The protein concentration ($Abs_{280}$) of sera samples was measured. Sera samples were then loaded onto a protein G column (Amersham Gamma-Bind G, 10 mg protein/ml bead). The bound antibody was washed with 3 column volumes of PBS pH 7.4 and then eluted with 2 column volumes of acetic

20  acid (0.1 M, pH 3.0). The elution peak was neutralized with Tris buffer (1 M, pH 9.0) (0.5 ml in 4 ml fraction) and then dialyzed back into PBS.


**Murine monoclonal IgGs specific for *E. coli* XL-1 blue**

At Day 0, 129 Gix+ mice (6-8 weeks, 4 per group) received *intraperitoneal*

25  injections of heat killed (65 °C, 15min), chemically competent *E. coli* XL-1 at $OD_{600}$=1 in a volume of 150 µl with 50 µl of phosphate buffered saline, PBS pH 7.4. At Day 14, the mice received a second injection in the same manner as the first. At Day 28, the mice received a third injection in the same manner as the first and second injections. At Day 35 mice were bled *via* intraocular puncture.

Twelve monoclonal antibodies specific for XL-1 blue were prepared using standard protocols. Antibody preparations were purified by ammonium sulfate precipitation followed by loading onto a protein G column (Amersham Gamma-Bind G, 10 mg protein/ml bead). The bound antibody was washed with 3 column

5     volumes of PBS pH 7.4 and then eluted with two column volumes of acetic acid (0.1 M, pH 3.0). The elution peak was neutralized with Tris buffer (1 M, pH 9.0) (0.5 ml in 4 ml fraction) and then dialyzed back into PBS.

**Generic ELISA for determining antibody-binding to live or killed *E. coli***

10     The $OD_{600}$ of a frozen glycerol stock of *E. coli* XL1-blue was read and the live bacterial stock was diluted in PBS to $OD_{600} = 1.0$. Twenty-five microliter aliquots of bacteria were placed in wells of a 96-well hi-bind ELISA plate and allowed to dry overnight at 37 °C. Plates were gently washed twice with dH2O. Plate wells were blocked with BLOTTO (50 µl/well) for 30 min at room

15     temperature and this blocking solution was removed by shaking. The antibody-containing sample to be assayed was then diluted into BLOTTO and 25 µl of this solution was placed in each well. Plates were incubated at 37 °C for 1 h in a moist chamber, washed with $dH_2O$ (10 x) and 25 µl of a secondary antibody (HRP-goat anti-rabbit conjugate, 1:2000) in BLOTTO was added to each well. Plates were

20     incubated at 37 °C for 1 h in a moist chamber and washed gently with $dH_2O$ (10 x). Developer substrate (50 µl/well) was added and the plates were read at 450 nm after 30 min.

Dead bacterial samples were also used for ELISA. These samples were handled in the same manner as above, but before addition and adherence to ELISA

25     microtiter plates, the *E. coli* are heat killed (65 °C, 15 min).

**Bactericidal Assays**

In a typical experiment, a culture of *E. coli* (in log phase growth, $OD_{600} = 0.2$-$0.3$) was repeatedly pelleted (3 x 3,500 rpm) and resuspended in PBS (pH 7.4).

The PBS suspended cells were then added to glass vials and cooled to 4 °C. Hematoporphyrin IX (40 µM) and antibody (20 µM) were added and the vials were either placed on a light box (visible light, 2.8 mW cm$^{-2}$) or in the dark at 4 °C and incubated for 1 h. Viability was determined by recovery of colony forming units

5      (CFUs) on agar plates. Each experiment was performed at least in duplicate.

## Microscopy Studies

Samples were prepared for electron microscopy as follows. Cells were fixed with paraformaldehyde (2 % w/v), glutaraldehyde (2.5 % w/v) in cacodylate (0.1 M)

10     at 0 °C for 1.75 h and then pelleted. The cell pellet was resuspended in $OsO_4$ (1% w/v) in cacodylate (0.1 M), allowed to stand for 30 min and then pelleted. The pellet was then sequentially dehydrated with ethanol and propylene oxide, embedded in resin and then sectioned. The sections were stained with uranyl acetate and lead citrate. For gold labeling studies, the procedure used was as detailed above with the

15     addition of the following steps. First, samples were pelleted and washed with fresh isotonic buffer to remove unbound primary antibody. Second, the pellet was resuspended in a solution of goat anti-mouse antibody that had been covalently modified with 12 nm gold particles, and incubated for 90 min.

20     ## Decomposition of $O_3$ Under Aqueous Conditions

The rate of decomposition of $O_3$ under the aqueous conditions employed was measured by the following method. Ozone, produced by a passage of $O_2$ through a Polymetrics ozonizer, was bubbled for 2 min through a phosphate buffered saline (PBS, pH 7.4) solution in a quartz cuvette (1 cm$^2$) at room temperature. The time-

25     dependent change in optical density was then measured at 260 nm ($\varepsilon$ = 2,700 M$^{-1}$ cm$^{-1}$) for at least 5 half lives in a Hitachi u.v./vis spectrophotometer equipped with a thermostatted rack at 22 °C. See Takeuchi et al., *Anal. Chim. Acta.* **230**, 183 (1990). The half-life of $O_3$ was then determined graphically ($t1/2$ = 66 sec) from a plot of OD vs. time using Graphpad Prism V 3.0 software (data not shown). The sensitivity

of the assay was limited by spectrophotometer accuracy to ± 0.1 % (~ 1 μM) of the OD at $t = 0$.

## Assay for Ozone

In a typical experiment, a solution of indigo carmine **1** (1 mM) in PBS (pH 7.4) was irradiated on a transilluminator (312 nm, 0.8 mWcm$^{-2}$) at room temperature in the presence or absence of antibody (20 μM) with or without catalase (13 mU/mL) in a quartz microtiter plate (final volume 200 μL), in duplicate. At various time-points a sample is removed (20 μL) and quenched into phosphate buffer (100 mM, pH 3.0, 180 μL). The OD was measured at 610 nm in a microtiter plate reader (Spectramax). Production of isatin sulfonic acid **2** was determined by LC-MS (Hitachi D-7000 HPLC linked to a Hitachi M-8000 ion-trap electrospray mass-spectrometer (in the negative-ion detection mode). LC conditions were a Spherisorb RP-C18 column and acetonitrile water (30:70) mobile phase at 1 mL/min. An in-line splitter was used to divert 0.2 mL/min of column effluent into the MS. Isatin sulfonic acid **2** RT = 3.4 min, [MH]- 226.

A variety of reactive species were tested to ascertain whether indigo carmine **1** could be converted to isatin sulfonic acid **2** by species other than ozone.

**Table 2:** Observed oxidation of indigo carmine **1**[a] and [18]O isotope incorporation into cyclic α-ketoamide **2**[b] by different reactive oxygen species.

| oxidant | Reaction to form 2 | [18]O incorporation into 2 |
|---|---|---|
| $O_3$[c] | Yes | yes |
| [1]$O_2$*[d] | Yes | no |
| $H_2O_3$[e] | Yes | no |
| $HO_2•/O_2•$[f] | No | -[h] |
| $H_2O_2$[g] | No | -[h] |
| $HOCl$[i] | No | -[h] |

[a]Oxidation was determined by following the absorbance change at 610 nm in a microtitre plate reader before and after addition of the respective oxidant to indigo

carmine **1** (1 mM) in phosphate buffer (PB, pH 7.4) at room temperature under the conditions specified.

[b]$^{18}$O incorporation was determined by performing the oxidation of indigo carmine **1** in PB (100 mM, pH 7.4) with $H_2$ $^{18}$O (>95% labeled) under the conditions specified

5    for each oxidant and monitoring the isotopic profile of cyclic α-ketoamide **2** by negative ion electrospray mass spectrometry. Under the conditions of the assay the label installed into the amide carbonyl of α-ketoamide **2** does not exchange with water.

[c]Indigo carmine (**1**, 1 mM) was added to a solution of ozone (~ 600 μM) in PB (100

10   mM, pH 7.0).

[d]The effect of $^1$O$_2$* was investigated by irradiation of an hematoporphyrin IX (40 μM) solution and **1** (1 mM) in PB with visible light (2.7 mW/cm$^{-2}$) for 1 h.

[e]See ref. 42.

[f]Potassium superoxide (10 mM) in DMSO was added to a solution of **1** in PB (100

15   mM, pH 7.0) such that the final organic cosolvent was 5 %.

[g]Final concentration 2 mM in PB.

[h]Not determined.

[i]Indigo carmine (**1**, 1 mM) was added to a solution of NaOCl (20 mM) in PBS (pH 7.4) and formation of cyclic α-ketoamide **2** was determined by HPLC after

20   complete bleaching of the solution occurred.

Preliminary studies revealed that, rapid and reversible exchange of the oxygen of the lactam carbonyl of cyclic α-ketoamide **2** with water occurred in the presence of u.v. light (312 nm, 0.8 mW cm$^{-2}$). However, in white light no

25   discernable exchange occurred during the experiment. Thus, all $^{18}$O isotope incorporations experiments were carried out using hematoporphyrin IX (40 μM) and white light (2.7 mW cm$^{-2}$) as the $^1$O$_2$* source.

Further studies were performed using the following additional chemical probes that contained a normal carbon-carbon double bond.



3-vinyl **3**
4 vinyl **4**

3-carboxy **5a**
4-carboxy **5b**

3-oxiranyl **6a**
4-oxiranyl **6b**
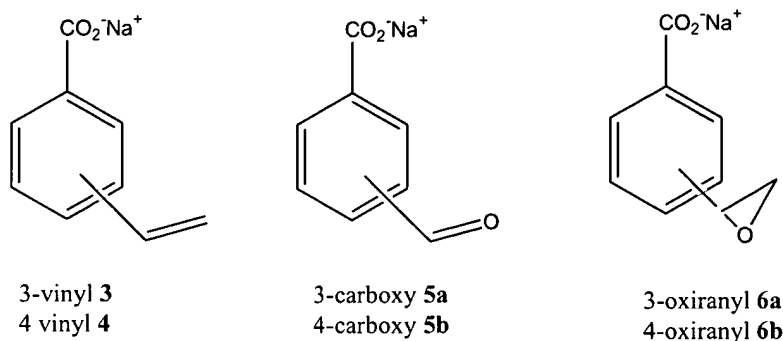
5      The choice of the probes, 3- and 4-vinyl-benzoic acid (**3** and **4** respectively), was guided by their aqueous solubility coupled with ease of detection by HPLC. In a typical experiment, a solution of 3-vinyl benzoic acid **3** (1 mM) or 4-vinyl benzoic acid **4** (1 mM) in PBS (pH 7.4) was irradiated (312 nm, 0.8 mW/cm$^{-2}$) at room temperature in the presence or absence of antibody 4C6, or sheep polyclonal

10    antibody (20 μM). Timed aliquots were removed (20 μL) and diluted 1:3 into acetonitrile:water (1:1). Product composition was determined by reversed-phase HPLC.

Conventional ozonolysis of 3-vinyl benzoic acid **3** (1 mM) in PBS (pH 7.4) at room temperature leads to the production of the benzaldehyde derivative **5a** with

15    minor production of the corresponding epoxide **6a** in a ratio of ~ 10:1. Similarly, ozonolysis of **4**, under the same conditions as described above, leads to 4-carboxybenzaldehyde **5b** and the corresponding oxirane **5b** in a ratio of ~ 9:1. In a typical experiment, a solution of **3** or **4** (1 mM) in PBS (pH 7.4) was added to a solution of $O_3$ in PBS (600 μM) at room temperature and allowed to stand for 5-10

20    min. The ozonolysis of **3** and **4** was performed in this manner rather than by bubbling an $O_3/O_2$ mixture through the aqueous reaction solution to prevent further oxidation of **3** and **4** that leads to hydroxylation and fragmentation of the aromatic ring. The product mixture and substrate conversion was elucidated by reversed-

phase HPLC. HPLC analysis was performed on a Hitachi D-7000 machine with a
Spherisorb RP-18 column and a mobile phase of acetonitrile and water (0.1 %
TFA)(30:70) at a flow rate of 1 mL/min. Localization was performed by u.v.
detection (254 nm) ($RT$ **3** = 7.84 min; $RT$ **5a** = 4.02 min; $RT$ **6a** = 3.82 min; $RT$ **4**

5    8.50 min;; $RT$ **5b** = 3.72 min; $RT$ **6b** = 4.25 min). Peak areas were converted to
concentration by comparison to standard curves.


**Antibody Detection on Neutrophils**

Neutrophils are known to have antibodies on their cell surface. Fluorescence

10    activated cell sorting (FACS) was used to measure the number of immunoglobulin
molecules per cell present under resting and activated conditions. Under resting
conditions there are approximately 50,000 antibody molecules per cell, which
increased to approximately 65,000 antibody molecules per cell upon activation.


15                                           **Results**

**Antimicrobial Activity of Antibodies**

As illustrated above, antibodies catalyze the generation of hydrogen
peroxide ($H_2O_2$) from singlet molecular oxygen ($^1O_2$*) and water by a process that
proceeds *via* dihydrogen trioxide ($H_2O_3$) intermediate. Results provided in this

20    Example illustrate that antibodies can utilize this process to efficiently kill bacteria.

Initial bactericidal studies utilized two strains of the gram-negative bacteria
*E. coli* (XL1-blue and O-112a,c). *E. coli* XL1-B was obtained from Stratagene. *E.
coli* O112a,c (ATCC 12804) is an enteroinvasive strain which can infect
malnourished and immuno-compromised individuals. Siegfried et al., *J. Med.*

25    *Microbiol.* **41**, 127 (1994).

The $^1O_2$* ion has bactericidal action. Berthiaume et al., *Biotechnology* **12**,
703 (1994). However, initiation of $H_2O_2$ production by antibodies requires
exposure to the substrate $^1O_2$*. Wentworth et al., *Proc. Natl. Acad. Sci. U.S.A.* **97**,
10930 (2000). Therefore, a $^1O_2$* generating system was used that would not, on its

own, kill *E. coli*. Antibodies can utilize $^1O_2^*$ generated by either endogenous or exogenous sensitizers or chemical sources, using u.v. or white light, or thermal decomposition of e.g. anthracene-9,10- dipropionic acid endoperoxide respectively. Therefore, the choice of a $^1O_2^*$ generating system is guided solely by experimental

5   considerations such as reaction efficiency and cellular or substrate sensitivity to irradiation. In these experiments, hematoporphyrin IX (HPIX, 40 µM) was selected as an efficient sensitizer of $^3O_2$. Wilkinson et al., *J. Phys. Chem. Ref. Data* **22**, 113 (1993). When irradiated with white light (light flux 2.7 mW cm$^{-2}$) for 1 h in phosphate buffered saline (PBS, pH 7.4) at 4 ± 1 °C, hematoporphyrin IX has

10  negligible bactericidal activity against the two *E. coli* serotypes (~ 107 cells/mL).

In a typical experiment, a culture of *E. coli* (in log phase growth, $OD_{600}$ = 0.2-0.3) was repeatedly washed in PBS by pelleting (3 x 3,500 rpm) the cells and resuspending them in PBS (pH 7.4). The PBS suspended cells were then added to glass vials and cooled to 4 °C. Hematoporphyrin IX (40 µM) and antibody (20 µM)

15  were added and the vials were either placed on a light box (visible light, 2.8 mW cm$^{-2}$) or in the dark at 4 °C and incubated for 1 h. Viability was determined by recovery of colony forming units (CFUs) on agar plates. Each experiment was performed at least in duplicate.

Addition of monoclonal antibodies (20 µM) to a mixture of

20  hematoporphyrin IX and bacteria resulted in killing of > 95 % of the bacteria (FIG. 14A). The bactericidal activity of antibodies was a function of antibody concentration. For example, killing of > 95 % of O112a,c cells was achieved with 10 µM of the antigen-specific murine monoclonal antibody 15404. These data indicate that the effective antibody concentration that kills 50 % of the cells ($EC_{50}$)

25  was 81± 6 nM (FIG. 14B). A similar concentration vs. kill dependence was observed for a specific monoclonal antibody (25D11) against the XL1-blue *E. coli* strain, with maximum killing > 95 % being observed at about 10 µM.

Antibody-mediated bactericidal activity increased both as a function of irradiation time (FIG. 14C) and with increasing hematoporphyrin IX concentration

(the light flux was fixed at 2.7 mW cm-2) (FIG. 14D). The observation that antibody-mediated bacterial killing is proportional to both hematoporphyrin IX concentration and light irradiation indicated that both $^1O_2*$ and the water oxidation pathway have a key role in the process. Critically, in the absence of $^1O_2*$,

5    immunoglobulins have a negligible effect on the survival of *E. coli*.

Controls indicated that cold shock and hematoporphyrin IX toxicity were not responsible for an appreciable loss of colony forming units (CFUs). Furthermore, confocal microscopy revealed that antibody mediated bacterial cell aggregation was also not contributing to a lack of CFUs in the antibody-treated groups. Fluorescence

10    analysis of the bacterial cells indicated that the amount of membrane-associated sensitizer in the hematoporphyrin IX-treated *E. coli* cells was not increased by antibody binding. Finally, while it is difficult to rule out the potential role of trace metals in the bactericidal action of antibodies, the presence of EDTA (2 mM) had no effect on the survival of bacteria in the assay system employed.

15    The bactericidal potential of antibodies appeared to be in general phenomenon. All twelve murine monoclonal antibodies (1 x κγ, 7 x κγ2a, 3 x κγ2b, 1 x κγ3 isotypes) and one rabbit polyclonal IgG (titer 120,000) sample that were tested were bactericidal. Nonspecific antibodies also were able to generate bactericidal agents. Only $^1O_2*$ was required for the activation of the water oxidation

20    pathway – such activation was independent of the antibody-antigen union. In this regard, 10 non-specific murine monoclonal antibodies, one non-specific sheep antibody preparation and one horse polyclonal IgG sample with no specificity for *E. coli* cell-surface antigens were studied and all possessed bactericidal activity. The potency of the bactericidal activity of antigen non-specific antibodies was observed

25    to be very similar to antigen-specific antibodies. Typically 20 µM of antibody (non-specific) was > 95 % bactericidal in the assay system. The bactericidal action of antibodies was not simply a non-specific protein effect as bovine serum albumin (BSA, 20 µM) exhibited no bacterial killing in the assay system.

To gain insight into the nature of the observed bacterial killing the

morphology of killed bacteria was studied by electron microscopy. Gold-labeled secondary antibodies were used to correlate the morphological damage to sites on the bacterial cell wall where antibodies were bound.

The killing is associated with the production of holes in the bacterial cell
5    wall at the sites of antigen-antibody union (FIG. 15). The process appeared to be a gradual one as evidenced by the range of morphologies present within the bacteria sampled. There were clear stages in the bactericidal pathway, in which oxidative damage led to an increased permeability of the cell wall and plasma membrane to water.

10    The bacterium is under an internal pressure of about 30 atmospheres, hence any weakening of the membrane can lead to catastrophic rupture. The process appeared to begin with slight disruptions observed at the interface between the cell wall and cytoplasm (FIG. 16A) that became more severe with clear separation of the cell wall from the cytoplasmic contents (FIG. 16B). Continued influx of water

15    resulted in gross distortion and deformity of the bacterial cell structure (FIG. 16C), ultimately leading to rupturing of the cell wall and plasma membrane and extrusion of the cytoplasmic contents at the sites of antibody attachment (FIG. 16D). In this regard, it is interesting that the observed morphologies induced by antibody-mediated killing are similar to those seen when bacteria are destroyed by

20    phagocytosis. Hofman et al., *Infect. Immun.* **68**, 449 (2000).


**The chemical nature of the bactericidal agents(s)**

If $H_2O_2$ was the ultimate product of the antibody-catalyzed oxidation of water pathway (Wentworth et al., *Proc. Natl. Acad. Sci. U.S.A.* **97**, 10930 (2000); P.

25    Wentworth, Jr. *et al Science* **293**, 1806 (2001)), then $H_2O_2$ alone would be the killing agent. This conclusion was strengthened by observations that catalase, which converts $H_2O_2$ to water ($H_2O$) and molecular oxygen ($O_2$), offered complete protection against the bactericidal activity of non-specific antibodies (FIG. 17A).

The amount of $H_2O_2$ generated by non-specific antibodies was $35 \pm 5$ µM.

The amount of $H_2O_2$ generated by specific antibodies was variable. The issue of proximity made a direct comparison between the effects of $H_2O_2$ in solution and $H_2O_2$ generated on the surface of the bacterial membrane complicated. For example, the protective effect of catalase (13 mU/mL) against the bactericidal

5     activity of 11 *E. coli* antigen-specific murine monoclonal antibodies and 11 *E. coli* non-specific murine monoclonal antibodies was studied. In all cases with non-specific antibodies, catalase completely attenuated the bactericidal activity. For the antigen-specific antibodies however, extent of protection by catalase was dependent on the monoclonal antibody used and varied over a wide range. Therefore,

10     proximity of $H_2O_2$ generation (directly on the surface of the bacterial membrane or in solution) affected the degree of protection offered by catalase. Hence, the effects of $H_2O_2$ in solution were compared only with $H_2O_2$ generated by antigen non-specific antibodies.

        The mean rate of $H_2O_2$ formation ($35 \pm 5$ µM/h) generated by non-specific

15     antibodies (20 µM) during the irradiation of a mixture containing hematoporphyrin IX (40 µM) with visible light (2.7 mW cm$^{-2}$) for 1 h at 4 °C in PBS (pH 7.4) was highly conserved. This mean value was determined from ten murine monoclonal IgGs and a sheep and horse polyclonal IgG ($n$=12).

        However, when the toxicity of $H_2O_2$ on the two *E. coli* cell lines was

20     quantified it became apparent that the amount of $H_2O_2$ generated by non-specific antibodies, $35 \pm 5$ µM, could not alone account for the potency of the bactericidal activity (FIG. 17B). This value was between 1 and 4 orders of magnitude below that required to kill 50 % of the bacteria, depending on whether the cell-line is XL1-blue or O112a,c respectively.

25     The combination of $H_2O_2$ with antibodies and/or $H_2O_2$ with hematoporphyrin IX was not more toxic to bacteria than $H_2O_2$ alone. These variables were tested to ascertain whether some interaction might occur between $H_2O_2$ and other components in the assay that would account for the potency of the bactericidal activity. In particular, the following combination of conditions were

tested for bactericidal activity against *E. coli* O112a,c:

1. $H_2O_2$ (2 mM) and non-specific antibody (20 μM);

2. $H_2O_2$ (2 mM) and antigen-specific antibody (20 μM); and

3. $H_2O_2$ (2 mM) and HPIX (40 μM).

5    Each group was irradiated for 1 h with visible light (2.7 mW cm$^{-2}$) at 4 °C. No enhancement in killing was observed for any of these combinations compared to that of $H_2O_2$ (2 mM) alone.

The finding that the toxicity of $H_2O_2$ to *E. coli* was below that generated by antibodies, necessitated re-examination of the experiments with catalase. One

10    possibility was that $H_2O_2$ reacted with some other chemical species that was also generated by the antibody, to produce other bactericidal molecule(s) and thus, by destroying $H_2O_2$, catalase prevented formation of that other chemical species. Another alternative was that the bactericidal species that were formed on the way to $H_2O_2$ was also a substrate for catalase.

15    Further experimentation indicated that ozone ($O_3$) was generated by antibodies. Under the aqueous conditions employed, ozone is quite long lived ($t1/2$ = 66 sec). Thus, ozone is sufficiently long lived to be detected by chemical probes such as indigo carmine **1**, a sensitive reagent for the detection of $O_3$ in aqueous systems. Takeuchi et al., *Anal. Chem.* **61**, 619 (1989); Takeuchi et al., *Anal. Chim.*

20    *Acta.* **230**, 183 (1990). Conventional ozonolysis of indigo carmine **1** in aqueous solution led to bleaching of the characteristic absorbance of indigo carmine **1** ($\gamma_{max}$ 610 nm, ε = 20,000 LM$^{-1}$cm$^{-1}$) and the formation of the cyclic α-ketoamide **2** (FIG. 18A).

To prove that ozone is produced by antibodies, the following experiments

25    were performed. A solution of indigo carmine **1** (1 mM) in PBS (pH 7.4) was irradiated with u.v. light (312 nm, 0.8 mW cm$^{-2}$) with no antibodies present. No bleaching was observed. However, when the same experiment was carried out in the presence of either a sheep polyclonal antibody (20 μM) or the murine monoclonal antibody 33F12 (20 μM) bleaching of indigo carmine **1** was observed

(FIG. 18B). Electrospray mass-spectrometry and HPLC analyses confirmed that cyclic α-ketoamide 2 was formed in this process. Sheep polyclonal antibody and monoclonal antibody 33F12 yield 4.1 μM and 4.9 μM of cyclic α-ketoamide 2 after 2 h of irradiation (312 nm, 0.8 mW cm$^{-2}$) of indigo carmine 1 (1 mM), respectively.

5    The initial rate of antibody mediated conversion of indigo carmine 1 into cyclic α-ketoamide 2 is linear, independent of the antibody preparation (sheep polyclonal IgG = 34.8 ± 1.8 nM min$^{-1}$, 33F12 = 40.5 ± 1.5 nM min$^{-1}$) (FIG. 18B).

The oxidative cleavage of the C=C double bond of indigo carmine 1 is a sensitive probe for ozone detection. Takeuchi et al., *Anal. Chem.* **61**, 619 (1989);

10    Takeuchi et al., *Anal. Chim. Acta.* **230**, 183 (1990). However, such cleavage was not specific for ozone. Further experiments with the oxidants listed in Table 2 were performed under the specified conditions to test whether those oxidants could also oxidize indigo carmine 1. Such experimentation confirmed that singlet oxygen ($^1O_2$) could bleach solutions of indigo carmine 1 to form cyclic α-ketoamide 2 by

15    oxidative double bond cleavage. $^1O_2$* is generated by antibodies upon u.v.-irradiation. Wentworth et al., *Proc. Natl. Acad. Sci. U.S.A.* **97**, 10930 (2000); Wentworth et al., *Science* **293**, 1806 (2001). An analytical differentiation between oxidative cleavage of indigo carmine 1 to cyclic α-ketoamide 2 by $^1O_2$* versus one by $O_3$ was therefore sought.

20    Further experimentation indicated that cleavage by $O_3$ could be distinguished from cleavage by $^1O_2$* by observing $^{18}O$ incorporation into the lactam carbonyl groups of cyclic α-ketoamide 2 when ozone is the oxidant. No such $^{18}O$ incorporation into the lactam carbonyl group of cyclic α-ketoamide 2 occurred when $^1O_2$* was the oxidant. Isotope incorporation experiments were therefore

25    carried out in $H_2^{18}O$ (> 95 % $^{18}O$) containing phosphate buffer (PB, 100 mM, pH 7.4) (Table 2 and FIG. 19), with the $^1O_2$* being generated by irradiation of hematoporphyrin IX (40 μM) with visible light (2.7 mW cm$^{-2}$). Preliminary experiments established that in $^{18}O$-water both indigo carmine 1 and 2 undergo slow but spontaneous isotope incorporation into the ketone-carbonyl groups of indigo

carmine **1** as well as of **2**, but *not* into the lactam carbonyl group of **2**. Thus, the diagnostic marker in the mass spectrum of **2** was the [M-H]- 230 fragment resulting from double isotope incorporation corresponding to $^{18}O$ incorporation into both the ketone and lactam carbonyl groups of **2**. Hence, in the mass spectrum of the

5  oxidation product, the mass peak [M-H]-230 was observed when the oxidation of indigo carmine **1** was carried out in $H_2^{18}O$ by chemical ozonolysis (FIG. 19B), but not when indigo carmine **1** was oxidized by $^1O_2*$ (FIG. 19C). *See* Gorman et al., in *Singlet Oxygen Chemistry*, 205 (1988).

When indigo carmine **1** (100 μM) was irradiated with visible light (2.8 mW

10  $cm^{-2}$) in the presence of sheep IgG (20 μM) and hematoporphyrin IX (40 μM), oxidized product **2** was formed that possesses a mass spectrum demonstrating exchange of $^{18}O$ of water into the lactam carbonyl (FIG. 19A). These data indicate that ozone was an oxidant for indigo carmine **1** when antibodies were present.

To further substantiate that ozone was generated by antibodies, the following

15  additional chemical probes that contained a normal carbon-carbon double bond were tested.



3-vinyl **3**
4 vinyl **4**

3-carboxy **5a**
4-carboxy **5b**

3-oxiranyl **6a**
4-oxiranyl **6b**
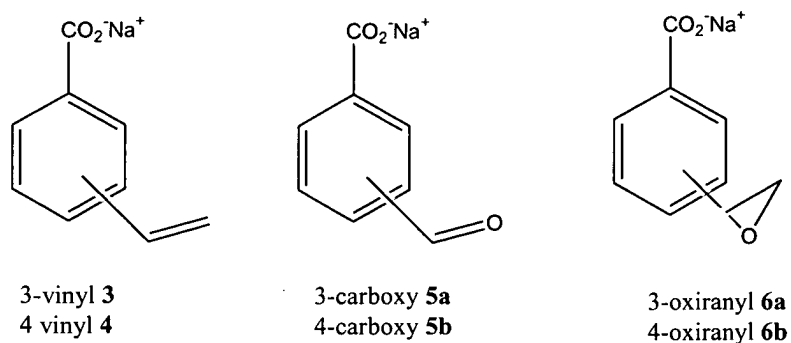
In a typical experiment, a solution of 3-vinyl benzoic acid **3** (1 mM) or 4-vinyl

20  benzoic acid **4** (1 mM) in PBS (pH 7.4) was irradiated (312 nm, 0.8 $mW/cm^{-2}$) at room temperature in the presence or absence of antibody 4C6, or sheep polyclonal antibody (20 μM). Timed aliquots were removed (20 μL) and diluted 1:3 into acetonitrile:water (1:1). Product composition was determined by reversed-phase

HPLC.

Irradiation of solutions of compounds **3** and **4** (1 mM) with u.v. light (312 nm, 0.8 mW cm$^{-2}$), in the presence of a sheep polyclonal IgG (20 μM), led to the formation of 3-carboxybenzaldehyde **5a** and 3-oxiranyl benzoic acid **6a** (ratio 15:1,

5    1.5 % conversion of **3** after 3 h) and 4-carboxybenzaldehyde **5b** and 4-oxiranyl-benzoic acid **6b** (ratio of 10:1, 2 % conversion to **4** after 3 h) respectively. These products are also observed when compounds **3** and **4** are ozonolized in a conventional way. Moreover, these results were similar to those observed for indigo carmine **1** irradiated with u.v. light in the presence of either a sheep polyclonal

10    antibody or the murine monoclonal antibody 33F12 where bleaching of indigo carmine **1** was observed (FIG. 18B). Again, if no antibodies were present, no bleaching was observed but in the presence of antibodies, oxidation products indicative of ozone were observed.

In sharp contrast, $^1O_2$* generated by hematoporphyrin IX (40 μM) and

15    visible light (2.7 mW cm$^{-2}$), did not cause any detectable oxidation of either **3** or **4** under similar conditions. Therefore, 3-vinyl benzoic acid **3** and 4-vinyl benzoic acid **4** are selective for ozone and the ozone must be produced by the antibodies present in the reaction.

20    **Evidence for Ozone Production by Activated Neutrophils**

Neutrophils are central to a host's defense against bacteria and are known to have antibodies on their cell surface and the ability, upon activation, to generate a cocktail of powerful oxidants including $^1O_2$*. Steinbeck et al., *J. Biol. Chem.* **267**, 13425 (1992); Steinbeck et al., *J. Biol. Chem.* **268**, 15649 (1993). Thus, these cells

25    therefore offer both a non-photochemical, biological source of $^1O_2$* and the antibodies capable of processing this substrate by the water-oxidation pathway. Most areas of the body do not have access to photochemical energy. Hence, if neutrophils provide a cellular source of $^1O_2$, an analysis of the oxidants expelled by antibody-coated neutrophils after activation could provide an indication as to

whether ozone or or $H_2O_2$ production by such antibodies may have a physiological relevance.

Human neutrophils were prepared as described by M. Markert, P. C. Andrews, and B. M. Babior *Methods Enzymol.* **105,** 358 (1984). Following

5     activation with phorbol myristate (1 μg/mL), the neutrophils (1.5 x $10^7$ cells/mL) produced an oxidant species that oxidatively cleaves indigo carmine **1** to isatin sulfonic acid **2** (FIG. 19 and FIG. 20B). While hypochlorous acid (HOCl) is an oxidant that is known to be produced by neutrophils, tests of NaOCl (2 mM) in PBS (pH 7.4) oxidized indigo carmine **1** (100 μM) but did not cleave the double bond of

10     indigo carmine **1** to yield isatin sulfonic acid **2**. When the oxidation of indigo carmine **1** was carried out in $^{18}O$ water, 50 % of the lactam carbonyl oxygen was found to consist of $^{18}O$, as revealed by the intensity of the [M-H]- 230 mass peak in the mass spectrum of the isolated cleaved product isatin sulfonic acid **2** (FIG. 20B). This $^{18}O$ incorporation indicates that ozone was generated by the antibody-coated

15     neutrophils.

FIG. 20A illustrates the time course of oxidation of indigo carmine **1** (30 μM) (▲) and formation of isatin sulfonic acid **2** (■) by human neutrophils (PMNs, 1.5 x $10^7$ cell/mL) that had been activated with phorbol myristate (1 μg/mL) in PBS (pH 7.4) at 37 °C. Interestingly, almost 50 % of the possible yield of isatin sulfonic

20     acid **2** (25.1 ± 0.3 μM of a potential 60 μM) from indigo carmine **1** was observed during the neutrophil cascade, revealing a significant concentration of the oxidant responsible for this transformation in the oxidative pathway.


**EXAMPLE IV: Microbicidal Action by Antibodies against *Salmonella***

25     This Example illustrates that antibodies directed against *Salmonella typhimurium* can kill those bacteria by generating reactive oxygen species.

## Methods

*Salmonella typhimurium* (ATCC 12804) was obtained from ATCC.

*Salmonella* non-specific murine monoclonal antibodies 33F12 and 84G3 were

obtained from the Scripps Hybridoma lab and used at > 98 % purity (based on SDS-

5  PAGE analysis).


### Murine Monoclonal Antibodies Generated for Bacterial Killing Studies

Gix+ mice that were 6-8 weeks old were used for generating antibodies

against heat killed (65 °C, 15min) *Salmonella typhimurium*. The following

10  immunization schedule was employed.

Day 0, 129 Gix+ mice (4 per group) each received an *i.p.* injection of heat

killed, chemically competent *Salmonella typhimurium* ($OD_{600}$=1) (150 $\mu l$ bacteria

and 50 $\mu l$ of phosphate buffered saline, PBS pH 7.4).

Day 14, the mice received a second injection of the same bacterial solution.

15  Day 28, the mice received a third injection, the same as 1st and 2nd

injection.

Day 35, the mice are bled *via* intraocular puncture.

Monoclonal antibodies were prepared following these immunization

protocols using standard protocols. Purification of these antibodies involved

20  ammonium sulfate precipitation followed by loading onto a protein G column

(Amersham Gamma-Bind G, 10 mg protein/ml bead). The bound antibody was

washed with 3 column volumes of PBS pH 7.4 and then eluted with 2 column

volumes of acetic acid (0.1 M, pH 3.0). The elution peak was neutralized with Tris

buffer (1 M, pH 9.0) (0.5 ml in 4 ml fraction) and then dialyzed back into PBS.

25

### ELISA assay for antibody-binding to live or killed *Salmonella typhimurium*

**Live bacteria**: The $OD_{600}$ of a frozen glycerol stock of *S. typhimurium* was

used to assess bacterial cell concentration and the bacterial suspension was diluted

in PBS to $OD_{600}$ = 1.0. The diluted bacterial suspension was aliquoted (25 $\mu l$/well)

into a 96-well hi-bind ELISA plate and allow to dry overnight at 37 °C. The plate

was gently washed with distilled water two times and the well were blocked with

BLOTTO (50 µl/well) for 30 min at room temperature. The BLOTTO was removed

by shaking. Antibody-containing samples in BLOTTO (25 µl/well) were then

5      added and the plates were incubated at 37 °C for 1 h in a moist chamber. The wells

were washed ten times with distilled water and a secondary antibody (HRP-goat

anti-rabbit conjugate, 1:2000, 25 µl well) in BLOTTO was added. The plates were

then incubated at 37 °C for 1 h in a moist chamber and washed gently with distilled

water ten times. The developer substrate was then added (50 µl/well). Plates were

10     read at 450 nm after 30 min.


**Dead bacteria**: The same procedure as described above was used for dead

bacteria, but the bacteria are heat killed (65 °C, 15 min) prior to addition to the

ELISA plate.

15

**Bactericidal Assays**

In a typical experiment, a culture of *S. typhmurium* in log phase growth at

$OD_{600}$ = 0.2-0.3 was repeatedly pelleted (3 x 3,500 rpm) and resuspended in PBS

(pH 7.4). The PBS suspended cells were then added to glass vials and cooled to 4

20     °C. hematoporphyrin IX solution (40 µM) and antibody (20 µM) were added. The

vials were placed either on a light box (visible light, 2.8 mW cm$^{-2}$) or in the dark at

4 °C. Incubation was for 1 hour. Viability was determined by recovery of colony

forming units (CFUs) on agar plates. Each experiment was performed with at least

duplicate samples.

25                                    **Results**

A panel of *S. typhimurium*-specific murine monoclonal antibodies were

raised and tested for bactericidal activity. Each antibody examined was bactericidal,

exhibiting greater than 50% killing of *S. typhimurium* after one hour irradiation in

the presence of a hematoporphyrin IX solution (120 µM) so long as the antibody

was present at a concentration of greater than 5 µM (FIG. 21). Antibody-concentration studies revealed that the maximum efficiency of bactericidal activity was reached at about 20 µM antibody. For example, killing of greater than 95 % of bacteria cells was achieved with 20 $\mu$M 6B5 (see FIG. 21).

5      Controls indicated that cold shock and hematoporphyrin IX toxicity are not responsible for an appreciable loss of CFUs. Furthermore, confocal microscopy revealed that antibody-mediated bacterial cell aggregation was not contributing to a lack of CFUs in the antibody-treated groups. Fluorescence analysis of the bacterial cells indicated that the amount of membrane-associated sensitizer in the

10    hematoporphyrin IX-treated *E. coli* cells was not increased by antibody binding. The bactericidal action of antibodies was not a non-specific protein effect as bovine serum albumin (BSA, 20 $\mu$M) exhibited no bacterial killing in the assay. Finally, the presence of EDTA (2 mM) had no effect on the survival of bacteria in the assay system detailed above.

15

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The specific methods and compositions described herein are representative

of preferred embodiments and are exemplary and not intended as limitations on the scope of the invention. Other objects, aspects, and embodiments will occur to those skilled in the art upon consideration of this specification, and are encompassed within the spirit of the invention as defined by the scope of the claims. It will be

5     readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. The invention illustratively described herein suitably may be practiced in the absence of any element or elements, or limitation or limitations, which is not specifically disclosed herein as essential. The methods and processes

10     illustratively described herein suitably may be practiced in differing orders of steps, and that they are not necessarily restricted to the orders of steps indicated herein or in the claims. As used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "a host cell" includes a plurality (for

15     example, a culture or population) of such host cells, and so forth. Under no circumstances may the patent be interpreted to be limited to the specific examples or embodiments or methods specifically disclosed herein. Under no circumstances may the patent be interpreted to be limited by any statement made by any Examiner or any other official or employee of the Patent and Trademark Office unless such

20     statement is specifically and without qualification or reservation expressly adopted in a responsive writing by Applicants.

       The terms and expressions that have been employed are used as terms of description and not of limitation, and there is no intent in the use of such terms and expressions to exclude any equivalent of the features shown and described or

25     portions thereof, but it is recognized that various modifications are possible within the scope of the invention as claimed. Thus, it will be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations

are considered to be within the scope of this invention as defined by the appended claims.

The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

Other embodiments are within the following claims. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.